GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR RECEPTORS

The present application is a continuation-in-part of Application Serial No.
08/747,842, pending, which claims priority benefit of provisional application serial
numbers 60/006,619, filed November 13, 1995; 60/015,767, filed April 16, 1996;
60/021,965, filed June 27, 1996; 60/020,638, filed June 27, 1996; and 20/020,639, filed
June 27, 1996, all applications hereby incorporated by reference in their entireties.

FIELD OF THE INVENTION

The present invention relates to the identification of receptors for and functions of
GDNF, and cell lines expressing the receptors.

BACKGROUND OF THE INVENTION

Glial cell line-derived neurotrophic factor (GDNF) is a trophic polypeptide. It is a
disulfide bridge-linked homodimer of two 134-amino acids long glycosylated
polypeptides, with a molecular weight of approximately 25-30 kD for each monomer.
Prior to the molecular cloning of GDNF in 1993, investigators sought a trophic
polypeptide which would alleviate the neuronal loss associated with Parkinson's disease,
specifically dopaminergic neurons of the ventral mesencephalon. The survival of this
subpopulation of neurons has been known for some time to be promoted by soluble
factors present in the conditioned media of glial cell lines. It was from one of these cell
lines that the GDNF protein was initially isolated based upon its ability to promote
dopamine uptake in primary cultures prepared from embryonic ventral midbrain neurons
(Lin et al., 260 Science 1120, 1993). Subsequently, GDNF was shown to promote
survival of adult substantia nigra neurons *in vivo* following pharmacological treatments
and lesions that mimic Parkinsonian syndromes (Beck et al., 377 Nature 339, 1995;
Tomac et al., 373 Nature 335, 1995) Although GDNF was originally reported to be

highly specific for dopaminergic neurons, several other potent activities of this molecule have subsequently been demonstrated, including survival and phenotypic responses in facial and spinal motor neurons (Henderson et al., 266 Science 30 1062, 1994; Oppenheim et al., 373 Nature 344, 1995; Yan et al., 373 Nature 341, 1995),

5   noradrenergic neurons of the locus coeruleus (Arenas et al., Neuron, in press, 1995), cerebellar Purkinjie cells (Mount et al., 92 PNAS 9092, 1995), sympathetic and sensory neurons in peripheral ganglia (Trupp et al., 130 J. Cell Biol. 137, 1995) and for populations of peripheral neurons with target-derived and paracrine mode of action (Trapp, M. et. al., *J. Cell Biol.*, 130, 137-148 (1995); Pitchel, J., Sariola, H., Hoffer,

10   B. & Westphal, H. (unpublished observation); Buj-Bello, A., Buchman, V. L., Horton, A., Rosenthal, A.& Davies, A. M. *Neuron, 15,* 821-828 (1995). As many of these neurons are affected in neurodegenerative diseases, GDNF may have potent therapeutical applications. Particularly, exogenously administered GDNF maintains dopaminergic neurons of the substantia nigra in experimentally induced

15   Parkinsons disease in rodents (Beck et al. (1995) *Nature,* 373, 339-341; Tomac et al. (1995) Nature, 373, 335-339) and leads to functional recovery in Parkinsonian rhesus monkeys (Gash et al. (1996) *Nature,* 380, 252-255). GDNF treatment also rescues about half of the experimentally axotomized murine motoneurons (Oppenheim et al. (1995) *Nature,* 373, 344-346; Li et al. (1995) *Proc. Natl, Acad. Sci. U.S.A.,* 92, 9771-9775)

20   suggesting that GDNF may be used in treatment of motoneuronal diseases. The studies of the mechanism of GDNF action in normal and pathogenic conditions have been, however, basically hampered as its receptor was not known.

Based upon structural similarities (primarily seven conserved cysteine amino acid residues), GDNF appears to be a distant member of the transforming growth factor-beta

25   (TGF-13) superfamily of multifunctional cytokines, which includes TGF-βs, activins, bone-morphogenetic proteins (BMPs) and growth and differentiation factors (GDFS) (Roberts et al., 327 *Philos.Trans.R.Soc.Land.* 145,1990). TGF-β and related ligands are known to suppress proliferation in epithelial and immune cells, to function as morphogens

in early development, to induce ectopic expression of skeletal tissue, and to promote survival and differentiation of neurons. TGF-β superfamily proteins interact with numerous receptor subunits on the surface of responsive cells (Attisano et al., 1222 Mol.Cell Res. 71, 1994; Derynck, 19 Trends Biochem. Sci. 548, 1994). Different

5   receptor types have been described based on the molecular weights of affinity labeled complexes. Among these are the type I, type II and type III receptors, which represent binding proteins of 55kD, 70kD and 3OOkD, respectively. Type III receptors are abundantly expressed transmembrane proteoglycans of approximately 3OOkD with a short cytoplasmic tail, and are thought to function in recruitment of ligand to an oligomeric

10   receptor complex (Lopez-Casillas et al., 67 Cell 785, 1991). Indeed, a type III receptor is required on some cell lines for TGF-β2 binding to the signaling receptors. Type I and type II receptors are transmembrane proteins with an intracellular serine-threonine kinase domain and can therefore transmit downstream signals upon ligand binding (Attisano et al., 75 Cell 671, 1993; Derynck, 1994 *supra*). Type II receptors are constitutively

15   activated kinases which upon ligand binding recruit type I receptors to a signaling complex. In this complex, type I receptors are phosphorylated by type II receptors on a juxtamembrane domain rich in serine residues, this phosphorylation is thought to result in the activation of the ser-thr kinase activity of type I receptors and in downstream signaling (Wrana et al., 370 Nature 341, 1994). According to this model, TGF-β

20   superfamily proteins can not bind to type I receptors in the absence of type II receptors, although in some cases, type I receptors are necessary for efficient binding to type II receptors (Letsou et al., 80 Cell 899, 1995). Multiple cDNA clones of type I, II and III receptors for TGF-βS, activins and BMPs have been isolated by either expression or homology cloning, including seven mammalian type I receptors, four type II receptors

25   and one type III betaglycan receptor. Additional membrane proteins binding different members of this family include glycosylphosphatidyl inositol (GPS)-linked 150kD and 180kD proteins of unknown structure and function (MacKay and Danielpour, 266 J. Biol. Chem. 9907, 1991), and endoglin, a 180kD disulphide linked dimer which binds TGF-β1

but not TGF-β2.

The isolation and characterization of GDNF receptors is a prerequisite for the understanding of the full range of biological actions of GDNF and the signaling events that take place upon GDNF binding to responsive cells. Until now, progress in this area

5   has been hampered by the lack of cell lines responsive to GDNF, that is, cell lines comprising GDNF receptors.

SUMMARY OF THE INVENTION

Receptors for GDNF are disclosed herein, as are cell lines expressing the same.

10   Methods for identifying and isolating these receptors are also disclosed.

In one aspect, the present invention relates to isolated receptors which bind GDNF.

In another aspect, the present invention relates a method for determining compounds or compositions which bind GDNF receptors.

15   In yet another aspect, the present invention relates to methods for identifying homologs of GDNF by screening for compounds or compositions which have similar biological effects, such as tyrosine phosphorylation, increase in c-fos mRNA, and increases in cell survival.

In still another aspect, the present invention relates to methods for identifying

20   analogs of GDNF by screening for compounds or compositions which are antagonistic for the biological effects of GDNF, such as are listed above.

In a further aspect, the present invention relates to compounds having the sequence as set forth in SEQ ID NOS:2 and 9.

In yet a further aspect, the present invention relates to nucleic acids having the

25   sequence as set forth in SEQ ID NOS:5 and 10.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Binding of ^{125}I-GDNF to receptors on chick sympathetic neurons. (a)

Saturation steady-state binding of ^{125}I-GDNF to E10 embryonic chick sympathetic neurons. Data are expressed as mean ±SD of triplicate determinations. (b) Scatchard transformation of the data plotted in (a). (c) Hill transformation of the data plotted in (a) nH: Hill coefficient.

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Fig.2. Affinity labeling of GDNF receptors on chick sympathetic neurons. ^{125}I-GDNF was cross-linked to E10 embryonic chick sympathetic neurons, receptor complexes were fractionated by SDS/PAGE and visualized by gel autoradiography (middle lane). A doublet at 100kD and a 300kD complex are indicated by arrows. Excess cold GDNF

10   prevented cross-linking of ^{125}I-GDNF (right lane). For comparison, crosslinking of ^{125}I-TGF-β to mink lung epithelial cells MvILu is also shown (left lane). Molecular weight markers are indicated in kD.

Fig.3. Affinity labeling of GDNF receptors on cell lines. ^{125}I-GDNF was cross-linked

15   to C6 glioma, RN33B raphe nucleus, L6 myoblast and MN-1 motor neuron cell lines with either DSS or EDAC as crosslinker agents. Receptor complexes were fractionated by SDS/PAGE and visualized by gel autoradiography. Excess cold GDNF prevented cross-linking of ^{125}I-GDNF (cold). Molecular weight markers are indicated in kD.

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Fig. 4. Individual constituent affinities of GDNF receptor subunits in RN33B and MN-1 cells. (a) Sizes of different GDNF receptor complexes on RN33B and MN-1 cells after cross linking with EDAC or DSS. (b) and (c) ^{125}I-GDNF was cross-linked to RN33B (b) or MN-1 (c) cells in the presence of increasing concentrations of cold GDNF. The

25   percentage of ^{125}I-GDNF binding to the indicated receptor subunit is plotted as a function of the concentration of cold GDNF used during binding.

Fig. 5. Expression of GDNF mRNA in cell lines expressing GDNF receptors. (a)

Autoradiogram of an RNAse protection assay using equal amounts of total RNA from the indicated cell lines. Kidney post natal day 1 and yeast tRNA were used as positive and negative controls, respectively. (b) Quantification of the level of GDNF mRNA in different cell lines relative to the level in PI kidney. undiff RN33B, undifferentiated

5   RN33B cells; diff RN33B, differentiated RN33B cells; diff RN338+GDNF, RN33B cells differentiated in the presence of GDNF.

Fig. 6. Expression of mRNA for *c-ret* in different cell lines.

10   Fig. 7. GDNF stimulation of tyrosine phosphorylation of ERKs in RN33B and MN-1 cells. RN33B (a) or MN-1 (b) cell monolayers were exposed to 50 ng/ml GDNF during the indicated periods of time (in minutes), cell lysates were fractionated by SDS/PAGE and Western blots probed with an anti-phosphotyrosine antibody (aP-Tyr). The blots were stripped and reprobed with an anti ERK2 antibody (a-ERK2) that recognizes both p42^{erk}

15   12 and p44^{erk1} (arrows to the right). Molecular weight markers are indicated in kD.

Fig. 8. GDNF stimulation of c-fos mRNA expression in RN33B and MN-1 cells. RN33B (a) or MN-1 (b) cell monolayers were exposed to 50 ng/ml GDNF during the indicated periods of times, total RNA was extracted and fractionated in agarose gels and

20   Northern blots probed with a ^{32}P-labeled rat c-fos probe. Shown are x-ray autoradiograms of filters washed at high stringency.

Fig. 9. GDNF increased the survival of RN46A cells. RN46A cells were differentiated in media ± 0-50 ng/ml GDNF for 8 days. The data represent the means ± SEM of

25   three independent experiments (1,500-3,000 cells counted per condition). ANOVA indicated that the GDNF had a significant effect on survival at all concentrations compared to media alone (overall ANOVA: df=6,203; F=1 1.39, p,0.001; unequal N LSD post hoc test, p.0.001).

Fig. 10 a-c. Biological and biochemical responses of MN-1 to GDNF. (a) GDNF stimulates survival of serum-deprived MN-1 cells. (b)GDNF stimulates rapid and transient tyrosine phosphorylation of several proteins (asterisks) in MN-1 cells. Time of GDNF treatment (in minutes), and molecular weight markers are indicated. (c)

5   Rapid and sustained ERKI and ERK2 tyrosine phosphorylation stimulated by GDNF in MN-1 cells.

Fig.11 a-b. c-RET is a signal transducing receptor for GDNF. (a) Immunoprecipitation analysis of GDNF-receptor complexes in MN-1 cells. GDNF-

10   labeled binding   proteins could be precipitated with lectin Sepharose beads, or antibodies against GDNF, phospho-tyrosine (P-Tyr) and c-RET. Control preimmune antibodies did not immunoprecipitate GDNF receptor complexes. (b) GDNF induces tyrosine phosphorylation of c-RET in MN-l cells. c-RET tyrosine phosphorylation was detected already 5 minutes after addition of GDNF (upper panel). Saturation was

15   observed at 30 ng/ml GDNF (lower panel).

Fig. 12 a-b. c-ret expression is sufficient to mediate binding and biological responses to GDNF in fibroblasts. (a) Iodinated GDNF could be cross linked to 3T3 cells stably transfected with MEN2a-ret or wild type c-ret expression plasmids. Untransfected

20   3T3 cells (3T3) did not bind GDNF. The specificity of the binding was demonstrated by displacement of the labeling with 50X excess cold GDNF. (b) GDNF promotes survival and growth responses in 3T3 fibroblasts stably transfected with a c-ret expression plasmid. Untransfected cells did not respond to GDNF.

25   Fig. 13 a-c. c-ret mRNA expression in adult brain and in developing substantia nigra. (a) Ribonuclease protection analysis (RPA) of c-ret mRNA expression in different regions of the adult rat brain. (b) RPA of c-ret mRNA expression during development of the rat ventral mesencephalon (nigra), and of GDNF mRNA expression in the developing

striatum. (c) mRNA expression is indicated in arbitrary units where 100 corresponds to the level of expression in the respective regions in newborn animals.

Fig. 14 a-h. c-RET is expressed in GDNF-responsive substantia nigra dopaminergic
5   neuron. (a) Dark field autoradiogram of *c-ret* mRNA expression analyzed by in situ hybridization in the adult substantia nigra. Scale bar, 40 μm. (b) Bright field autoradiogram showing substantia nigra neurons containing *c-ret* mRNA. Scale bar, 7.5 μm. (c) Immunohistochemical analysis of c-RET protein expression in the adult substantia nigra. Scale bar, 27 μm. (d) autoradiogram showing *in situ* hybridization for *c-ret*
10   mRNA in the adult rat brain after a unilateral lesion with 6-OHDA. The injection of this toxic dopamine analogue in the medial forebrain bundle ensures that only cells which actively take up and retrogradely transport dopamine will be compromised. Note the disappearance of the labeling for *c-ret* mRNA in the lesioned substantia nigra (arrowhead) 1 day and 5 days following the lesion. (e)Immunohistochemical analysis of c-RET
15   protein expression in the adult substantia nigra after lesion with 6-OHDA and grafting of mock transfected fibroblasts (control graft). Note the nearly complete absence of c-RET-LI caused by the lesion. Scale bar, 20 μm. (f) Grafting of GDNF-expressing fibroblasts rescues c-RET-LI. Note c-RET positive fibers surrounding and entering the GDNF producing graft (arrows). Same magnification as in (e). (g) Immunohistochemical
20   analysis of cRET protein expression in the adult locus coeruleus after lesion with 6-OHDA and grafting of mock transfected fibroblasts (control graft). Scale bar, 23 μm. (h) Rescue of cell bodies expressing c-RET-LI by GDNF in of 6-OHDA lesioned locus coeruleus. Same magnification as in (g). Graft is on the right in (e) and (f), and above in (g) and (h).

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Fig. 15 a-c. PC12 and NB2/a cells respond to GDNF and bind GDNF. (a) GDNF promotes survival of serum-deprived PC12 cells. (b) GDNF increases the number of NB2/a cells. (c) ^{125}I-GDNF binds to PC12 and NB2/a cells in the absence (open column)

or presence (filled column) of 50-fold unlabeled GDNF.

Fig. 16. Affinity crosslinking of ^{125}I-GDNF to cell lines. ^{125}I-GDNF was crosslinked to PC12 cells (lane 1), SY5Y cells (lane 2), E20 rat kidney cells (lane 3) and NB2/a cells

5 (lane 4), and the resulting complexes were precipitated from detergent lysates by anti-GDNF antibodies (Santa Cruz).

Fig. 17 a-b. GDNF specifically binds to c-RET. (a) ^{125}I-GDNF was crosslinked to NB2/a cells in the presence (+) or absence (-) of 1000-fold excess of unlabeled GDNF

10 (PeproTech EC Ltd.), and the resulting complexes were precipitated from detergent lysates by cocktail of monoclonal and polyclonal (Santa Cruz) anti-c-RET antibodies recognizing the extracellular and intracellular domain of cRET, respectively. Lysates were also precipitated by monoclonal anti-neurofilament antibodies 13AA8 (lane 3), by Protein A-Sepharose (lane 4) and by WGA-Agarose (lane 5). (b) ^{125}I-GDNF binds to COS

15 cells transiently expressing c-RET, but not to mock-transfected (with pBK-CNV plasmid) COS cells. Open column represents binding in the presence, and filled column in the absence of 50-fold excess of unlabeled GDNF.

Fig. 18. GDNF increases tyrosine phosphorylation of c-RET in transfected COS cells. c-

20 RET was immunoprecipitated from detergent lysates of GDNF-treated (+) (lane 1) or untreated (-) (lane 2) COS cells transfected (lane 3) with c-ret cDNA or mock-transfected with PBK-CMV plasmid. (a) immunoblot probed with anti-c-RET antibodies (Santa Cruz). (b) the same filter reprobed with anti-phosphotyrosine antibodies.

25 Fig. 19 a-h. GDNF binds *in situ* to *c-ret*-positive developing enteric neurons. (a, b) Dark-field (a) and corresponding bright-field (b) microphotographs of GDNF antisense cRNA hybridization to paraffin sections through E15 rat gut. (c, d) Dark-field (c) and corresponding bright-field (d) microphotograph of *in situ* binding of ^{125}I-GDNF to E15 rat

gut explants. (e) c-*ret* antisense cRNA hybridization to a cryosection through E15 rat gut. (f) Immunostaining of E15 rat gut cryosection with anti-peripherin antibodies. (g) GDNF sense cRNA hybridization to E15 rat gut section. (h) *In situ* binding of ^{125}I-GDNF to E15 rat gut explants in the presence of 250-fold excess of unlabeled GDNF.   ---, muscle

5   layer; n, intestinal nerve plexus. Bar, 100μm.

Fig. 20 a - b. Crosslinked GDNF-c-RET-complexes are obtained from GDNF-responsive cell lines and from c-*ret*-transfected cells (a) ^{125}I-GDNF was crosslinked with EDAC to PC12 cells, NB2/a cells, dissociated E20 rat kidney cells, and COS cells, and the

10   resulting complexes were precipitated by anti-GDNF antibodies. (b) EDAC-crosslinked ^{125}I-GDNF-c-RET complexes were immunoprecipitated with anti-c-RET antibodies from the extracts of PC12 cells, stably c-*ret*-transfected (Ret.-3T3) or mock-transfected (mock-3T3) 3T3 cells, as well as from dissociated E15 kidney cells in the presence (+) or absence (-) of 500-fold excess of unlabeled GDNF or TGF-β1. The ~50K bands in all

15   gels are the crosslinked dimers of GDNF.

Fig. 21 a - b. GDNF increases c-RET autophosphorylation in stably transfected 3T3 cell line. (a) GDNF dose-dependently increases tyrosine phosphorylation of 160 kD isoform of c-RET in c-*ret*-transfected (ret-3T3) but not in mock-transfected (mock) cells. (b)

20   GDNF time-dependently increases tyrosine phosphorylation of 160 kD isoform of cRET in c-*ret*-transfected 3T3 cells. Upper panels (Ret.-PTyr) are the immunoblots stained with anti-phosphotyrosine antibodies, and lower panels (Ret.) show the reprobing of the corresponding filters with anti-c-RET antibodies.

25   Fig. 22. GDNF increases the number of trkC-3T3 fibroblasts transiently expressing c-RET (open squares), but not mock-transfected cells (filled squares). c-*ret* and mock-transfected cells in five parallels were treated with rat GDNF at indicated concentrations, or with NT-3, for five days. Cell number, quantified with Abacus™ Cell Proliferation Kit

(Clontech), is expressed as a percent of the control cells without growth factors. *, $p < 0.001$ compared to mock transfected cells.

Fig. 23 a-b. Purification of receptor from L6 myeloblast cells. (a) Plasmon resonance analysis of fractions obtained from anion exchange chromatography of L6 cell lysates. Total protein of fractions is also depicted. (b) Further purification of 1M fraction obtained from (a) by hydrophobic interaction chromatography.

Fig. 24. Autoradiographic film of the ligand blot ^{125}I-GDNF with proteins from adult rat brain (lane 2) and liver (lane 3). 50-fold excess of unlabeled GDNF (lane 1) significantly reduces the binding.

Fig. 25. Amino acid sequence of GDNFR-β.

Fig. 26 a-b. Comparison of amino acid sequences of a) rat GDNFR-α and GDNFR-β and b) rat and human GDNFR-α and GDNFR-β.

Fig. 27. Expression of GDNFR-α mRNA using RNAse protection analysis.

Fig. 28. Expression of GDNFR-β mRNA using RNAse protection analysis.

Fig. 29. Chemical cross linking of ^{125}I-GDNF to GDNFR-α and GDNFR-β in transfected COS cells.

Fig. 30. Comparison of complexes obtained upon cross linking of ^{125}I-GDNF to GDNFR-α and GDNFR-β in COS transfected cells with complexes obtained upon cross linking of ^{125}I-GDNF to receptors on RN33B cells.

Fig. 31. Effect of co-transfection of GDNFR-α and GDNFR-β with c-Ret in COS cells upon phosphorylation of c-RET (a) and effect of transfection of GDNFR-α and GDNFR-β in Neuro-2A cells upon phosphorylation of c-RET.

5   Fig. 32. The cDNA sequence for rat GDNFR-β.

Fig. 33. (a) The cDNA sequence of human GDNFR-β. The translation termination site and the sequences of the primers used to amplify the 5' end of the gene are marked in bold. The first and last 6 nucleotides of the sequence (nucleotides 469-1490) derived

10   from I.M.A.G.E. EST clones are underlined. (b) The alignment of the predicted human and rat GDNFR-β and GDNFR-α proteins. N-glycosylation sites are underlined and amino acid residues identical in at least three out of the four sequences are marked in bold.

15   Fig. 34. Northern blot showing the expression of GDNFR-β mRNA in multiple human tissues. The molecular weight marker sizes are the same in all the filters. The lower panels present the same filters rehybridized with human β-actin probe.

Fig. 35. Grayscale image of fluorescent *in situ* hybridization of the human (a) and mouse

20   (b) GDNFR-β genes on metaphase chromosomes.

Fig. 36. GDNFR-β mediates GDNF-induced c-Ret autophosphorylation in transiently transfected COS-7 cells.

25   Fig. 37. Bright and dark field images of consecutive sections through adrenal gland (A, B,C,), kidney (D, E, F), small intestine (G, H, I), spinal cord (J, K, L), and trigeminal ganglia (M, N, O) of E17 rat, hybridized with probes to rat GDNFR-β (B, E, H, K, N) or GDNFR-α (C, F, I, L, O). Arrows indicate low GDNFR-β mRNA expression in the

undifferentiated mesenchyme of kidney (E) and enteric neurons of gut (H).

Abbreviations: ac = adrenal cortex, am = adrenal medulla, drg = dorsal root ganglion, en = enteric nervous layer, mn = ventral motoneuron column, nt = neural trunk of trigeminal secretory tubules, tg = trigeminal ganglion, u = tip of ureter bud, vr =

5   ventral root of spinal cord. Bar = 200μm.

DETAILED DESCRIPTION

A prerequisite for the understanding of the full range and mechanisms of action of

10   GDNF is the characterization of GDNF receptors and their signaling pathways. Although receptors for other members of the TGF-β superfamily are well characterized, GDNF receptors remained undefined until this disclosure. Disclosed herein is the biochemical characterization of GDNF receptors and their downstream responses in sympathetic neurons and responsive cell lines. Using affinity labeling, multiple GDNF binding

15   subunits that mediate cooperative binding of GDNF to embryonic sympathetic neurons are identified. Screening of over thirty cell lines initially revealed high expression of GDNF binding proteins of 55 kD, 70kD, 135 kD and 300 kD in conditionally immortalized neuronal precursors from the raphe nucleus. As the data demonstrate, GDNF receptors were highly induced after neuronal differentiation of these cells, which

20   then became sensitive to the survival-promoting effects of GDNF. Different combinations of these subunits were also seen in glioma, myoblast and Sertoli cells. A different receptor pattern was found in a motor neuron hybrid cell line, where the predominant component was a CPI-anchored protein of 155kD.

Despite the striking similarity with the receptor pattern of other TGF-β

25   superfamily members, immunoprecipitation experiments indicated that GDNF receptor subunits of 55kD, 70kD, 135kD, and 300kD are novel proteins. The 55kD binding protein has been confirmed to be GDNFR-α described as being cloned from the substantia nigra (Treanor et al. *Nature*, 382:80-83, 1995) and from the retina (Jing et al.

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Cell, 85:1113-1124,1996). GDNFR-α is attached to the cell membrane by a glycosylphosphatidyl inositol linkage (GPI) and, thus, cannot transmit intracellular signals on its own. The 70 kD binding protein has now been cloned and sequenced from the rat and human and has been designated as "GDNFR-β."

5   We assign the locus of GDNFR-β to human chromosomes 8p21-22 and mouse chromosome 14D3-E1. The 155kD subunit was subsequently determined to be the product of the *c-ret* proto-oncogene, c-RET, a receptor tyrosine kinase crucial for the development of parts of the excretory and nervous systems. GDNF stimulated ERK tyrosine phosphorylation and c-fos mRNA expression with different time-courses in raphe

10   nucleus and motor neuron cell lines, suggesting that different complements of GDNF receptor subunits can form distinct signaling complexes.

Concomitantly, c-RET was identified as receptor for GDNF on additional cell lines. GDNF rescues c-RET-positive dopaminergic and noradrenergic neurons in lesion models of Parkinson's disease, suggesting that cRET may mediate the anti-Parkinsonian

15   effects of GDNF in the adult brain.

c-ret proto-oncogene (Takahashi et al. (1985) *Cell*, 42, 581-588) encodes a protein that is structurally related to receptor tyrosine kinases (Takahashi et al. (1988) *Oncogene*, 3,571-578). Its extracellular part contains an unusual cadherin-like domain and also a cysteine-rich domain, the biological roles for which are not understood. By alternative

20   splicing, several isoforms of c-*ret* mRNA have been described (Tahira et al. (1990) *Oncogene*, 5, 97-102; Myers et al., (1995) *Oncogene*, 11, 2039-2045;Lorenzo et al. (1995) *Oncogene*, 10, 1377-1383), but their biological meaning is currently not understood. In several cell lines, *c-ret*-encoded proteins with molecular weights of 160 kD and 140 kD are described, representing the fully and partially glycosylated isoforms

25   of 120 kD core protein, respectively (Takahashi *et al.*, 1988). As with other receptor tyrosine kinases, c-RET is activated by homodimerization followed by phosphorylation of its tyrosine residues.

In the excretory system, *c-ret* is expressed in the nephric duct, the ureteric bud

and the growing tips of the collecting ducts (Pachnis et al., (1993), *supra*). Mice homozygous for a null mutation in the *c-ret* gene die soon after birth, with kidneys either absent or rudimentary and displaying severe defects in the enteric nervous system (Schuchardt et al., *Nature* 367, 380-3 (1994). Based on this evidence, it had been

5   proposed that the cognate *c-ret* ligand may be a growth factor important for morphogenesis and neurogenesis.

During murine embryogenesis, *c-ret* mRNA is expressed primarily in the nervous and excretory systems. *c-ret* mRNA is found in dorsal root, sympathetic, enteric and cranial ganglia (Pachnis et al., *Development* 119, 1005-17 (1993), as well as in post

10   migratory neural crest cells and in various tumors of neural crest origin, including pheochromocytoma, medullary thyroid carcinoma and neuroblastoma (Ikeda, I., *et al. Oncogene 5*, 1291-6 (1990); Santoro, M., *et al. Oncogene 5*, 1595-1598 (1990). In the developing central nervous system, sites of *c-ret* expression include the ventral portion of the neural tube, the retina and motor neurons in spinal cord and hindbrain (Pachnis et al.,

15   (1993), *supra*). However, the pattern of expression of *c-ret* in the adult nervous system has not previously been reported.

The absence of a known ligand for c-RET has basically hampered the studies of intracellular pathways that c-RET can mediate. Comparative analysis of the growth-promoting activity of the epidermal growth factor receptor/c-RET chimera expressed in

20   fibroblastic or hematopoietic cells revealed a biological phenotype clearly distinguishable from that of epidermal growth factor receptor (Santoro et al. (1994) *Mol. Cell. Biol.* 14, 663-675). We disclose herein that both NGF and GDNF promote survival of PC12 cells, whereas only NGF induces their differentiation, suggesting only a partial overlap in the signaling pathways of c-RET and trkA, a receptor for NGF. Binding of an adaptor

25   protein Grb2 to oncogenic forms of c-RET has been demonstrated (Borrello et al. (1994) *Oncogene*, 9, 1661-1668). However, the details of the pathways are completely unknown. Now, having GDNF as a ligand, it is possible to address the intracellular signaling of c-RET upon GDNF binding.

Like c-RET, GDNF is abundantly expressed in the muscle layer of the
gastrointestinal tract and in the condensing mesenchyme of the kidney (Suvanto et al.
(1996) *Eur. J. Neurosci.*, 8, 816-822). Further, as disclosed herein, GDNF specifically
binds c-RET-positive cells in developing gut, GDNF can be cross linked to c-RET in
5   several cell lines and in developing kidney, GDNF specifically induces tyrosine
phosphorylation of c-RET, and ectopical expression of c-RET in 3T3 cells confers a
biological response of these cells to GDNF. Thus, c-RET is activated by GDNF and
mediates its functions.

As disclosed further herein, GDNFR-α and GDNFR-β mediate GDNF-induced c-
10   RET autophosphorylation in transfected cells. The presence of the similarly behaving
GDNF presenting proteins may lower the amount of GDNF needed to activate c-RET.
By Northern hybridization, we disclose that the transcript level of human GDNFR-β
mRNA is high in the adult brain, intestine and placenta and in fetal brain, lung and
kidney. Studied by *in situ* hybridization, GDNFR-β mRNA shows in E17 rat embryo
15   different distribution than that of GDNFR-α mRNA, especially in adrenal gland, kidney
and gut. In the developing nervous system, GDNFR-β mRNA expression is restricted to
certain neuronal populations, while GDNFR-α mRNA is widely expressed also in non-
neuronal cells. The distinct tissue distribution of GDNFR-β mRNA and its ability to
mediate GDNF signal in transfected cells suggest a role in signal transduction of GDNF
20   and, possibly, related neurotrophic factors *in vivo*. The fact that GDNFR-β mRNA is
present in some organs (such as adrenal cortex) where GDNF is not available points out
to a possibility that some other ligand (like GDNF homolog such as neurturin and
persefin; Kotzbauer et al., *Nature*, 384:467-470, 1996 and Kotzbauer et al.,
Differentiation and Degeneration, Keystone Symposia on Molecular and Cellular Biology,
25   Taos, NM, March 27 - April 2, 1996, p. 136, respectively) may use GDNFR-β in their
signal transduction. Likewise GDNFR-β may also be used in the activation of other
signaling receptors than Ret.

GDNF and the genes responsible for its signal transduction are of great clinical

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interest due to their potential use in therapy for motoneuron and Parkinson's diseases. In addition, these genes are intensively studied as possible candidate disease genes for congenital or inherited disorders affecting the survival of the neurons in substantia nigra and in the gastrointestinal tract.

5   The product of the *c-ret* proto-oncogene plays important roles in human disease. Rearrangements and mutations in the *c-ret* gene are associated with several tumors e.g. familial medullary thyroid carcinoma, multiple endocrine neoplasia type 2, etc., but also with Hirschsprung disease, a disorder that is characterized by the absence of enteric neurons in the hindgut, resulting in obstipation and megacolon in infants and adults

10   (reviewed in Mak, Y. F. and Ponder, B.A. J. (1996)*Curr. Op. Genet. Dev.*, 6, 82-86). Identification of GDNF as a ligand for c-RET further enables the analysis of the molecular basis of these diseases. Particularly, the mutations in GDNF gene can now be studied as possible cause for the Hirschsprung disease in the cases where *c-ret* locus is not mutated.

15   At present, there is no candidate disease assigned to the human locus of GDNFR-β gene i.e., 8p21-22, but since it probably participates in the signal transduction of GDNF in neurons, the new receptor GDNFR-β is likely to be of great interest in investigations concerning neurodegenerative diseases. In addition, the gene for GDNF-β is a potent candidate disease gene for congenital disorders that resemble the phenotypes

20   of GDNF or RET Knock-out mice (e.g. Hirschsprung disease, kidney aplasia and dysplasis), and we are screening for mutations in these developmental orders.

        The phrases "GDNF receptor" and "receptor for GDNF" as used herein each refer to a single subunit which binds GDNF as well as combinations of the receptor subunits which bind GDNF.

25   The term "effect" as used herein means an alteration or change. An effect can be positive, such as causing an increase in some material, or negative, e.g., antagonistic or inhibiting.

        The term "homolog" as used herein refers to a compound or composition having a

similar biological effects as GDNF, such as are disclosed herein.

The term "analog" as used herein refers to a compound or composition having an antagonistic effect on the biological effects of GDNF.

The term "isolated" as used herein in reference to a GDNF receptor means a

5   compound which has been separated from its native environment or, if recombinantly expressed, from its expression environment.

The phrase "substantially pure" as used herein in reference to a compound means an isolated compound which has been separated from other components which naturally accompany it. Typically, a compound is substantially pure when it is at least 75%, more

10   preferably at least 90%, and most preferably 99% of the total material as measured, for example, by volume, by wet or dry weight, or by mole percent or mole fraction.

The phrase "non-permissive culture conditions" as used herein refers to conditions which do not normally support survival of the cells being cultured *in vitro*, e.g., temperature, media components, etc.

15   The phrase "an excess" as used in reference to the addition of labeled GDNF in a competitive assay refers to an amount of labeled GDNFsufficient to facilitate the detection of a competing compound -- for example, an amount of labeled GDNF which is twice the amount of the compound to be tested.

The term "bind" as used herein refers to the interaction between the GDNF ligand

20   and its receptor, the binding being of a sufficient strength and for a sufficient time to allow the detection of said binding under the conditions of the assays disclosed herein.

The term "about" in reference to a numerical value means ±10% of the numerical value, more preferably ±5%, most preferably ±2%.

Any claims to sequences herein encompass those insubstantial alterations which

25   can be made to a sequence without effecting function, i.e., substantially the same sequences. For example, a change in a nucleotide within a codon that results in the same amino acid as originally encoded by the codon is "substantially the same sequence." Also, a conservative amino acid substitution within the sequence that does not affect

function is also "substantially the same sequence."

The specific examples presented below demonstrate that:

1) GDNF receptor is present in multiple neuronal and non-neuronal cell types;

2) GDNF receptor is composed of multiple subunits which cooperate to achieve

5   high affinity binding;

3) members of the ERK/MAP kinase family are components of the GDNF

signaling mechanism; and

4) c-RET is a functional receptor for GDNF.

10   1. *Novel GDNF receptor expression* in *multiple neuronal and non-neuronal cell types*

Heretofore unidentified receptors are identified herein as GDNF receptors. These

novel GDNF receptors were found in cells lines of different origins, although they

appeared to be most abundant in neuronal cells. Preferably, the cell lines are selected

from the group consisting of RN33B, RN46A, and C6 (see Table I), with RN33B being

15   most preferred. The identification of GDNF receptors in many of these cell types

suggests novel cellular populations responsive to GDNF *in vivo*. GDNF has been shown

to promote survival and phenotype of distinct subpopulations of neurons, in particular

dopaminergic and noradrenergic central neurons, as well as spinal and facial motor

neurons. Given the activities of GDNF in various monoaminergic neurons, the discovery

20   of GDNF receptors in cell lines derived from the medullary raphe indicate that

serotonergic neurons may also respond to GDNF *in vivo*. The endogenous expression of

GDNF by these cells suggests that this factor may act in a paracrine/autocrine fashion

within the raphe nucleus. Expression of GDNF receptors in Sertoli TM4 cells suggests

non-neuronal roles for GDNF in developing testis. *In vivo*, the temporal expression of

25   GDNF mRNA in testis correlates with the expansion of the Sertoli cell population (Trupp

et al., *supra*) which, together with the discovery of GDNF receptors on the TM4 cell

line, suggest an autocrine action of GDNF during Sertoli cell maturation. Similarly, the

presence of GDNF receptors in rat myoblast L6 cells, together with the expression of in

developing muscle *in vivo* (Henderson et al., *supra;* Trupp et al., *supra*), indicates a potential paracrine role of GDNF during myogenesis. Despite the presence of receptors and biological activities of GDNF on embryonic sympathetic neurons, PC12 cells which had been differentiated into sympathetic-like neurons with NGF did not express GDNF

5   receptors under initial experimental conditions. As discussed below, however, GDNF receptors were ultimately identified on PC12 cells.

GDNF receptors are absent in the pons noradrenergic cell line CATH.a. Given the robust effects of GDNF on adult central noradrenergic neurons from the locus coeruleus, the absence of GDNF receptors in CATH.a is intriguing. Recently, however,

10   Gong et al. reported that GDNF can prevent the degeneration of CATH.a cells induced by 6-OH-dopamine treatment (Gong et al., 21 *Abs. Soc. Neurosci.*, 1789, 1995), suggesting that GDNF receptors may be induced in these cells after 6-OH-dopamine lesion. Indeed, *in vivo* studies have shown that GDNF elicits a more profound induction of the phenotype of noradrenergic neurons following 6-OH-dopamine injection than in the

15   non-lesioned locus coeruleus. Further, Treanor et al. recently reported upregulation of GDNF binding in sections of the substantia nigra after medical forebrain bundle transaction (Treanor et al., 21 Abs. Soc. Neurosci. 1301, 1995), suggesting that the receptor upregulation may be a general mechanism of control of GDNF responsiveness in the central nervous system.

20   GDNF receptor upregulation was also observed during *in vitro* differentiation of raphe nucleus cells. These lines have recently been shown to retain the ability to respond to local microenvironmental signals after transplantation into the adult brain, where they differentiate in a direction that is consistent with that of endogenous neurons in the transplantation site (Shihabuddin et al., 15 *J. Neurosci.* 6666, 1995). *In vitro,*

25   however, a shift to the non-permissive temperature differentiates them along default pathways into glutamatergic (RN33B) or serotonergic (RN46A) phenotypes, respectively. Differentiation in culture has also been shown to upregulate expression of receptors for other trophic factors in these cells, including the neurotrophin receptors $p75^{LNGFR}$ and

trkB (Whittemore and White, 615 British Res. 27, 1993). Although they can give rise to different neuronal types depending upon the site of transplantation, RN33B cells are not able to generate glial elements, suggesting these cells represent neuronally restricted multipotent precursors (Shihabuddin et al., *supra*). In this respect, it is interesting to note

5   the absence of GDNF receptors in two pluripotent neuronal stem cell types (Renfranz et al., 66 Cell 713, 1991; Snyder et al., 30 68 Cell 33, 1992) suggesting that these cells are less restricted than the raphe nucleus cell lines. Taken together, these observations suggest that GDNF receptor expression may initially appear in newly differentiated post-mitotic neurons and increase progressively during neuronal maturation.

10

2.   *Multiple GDNF receptor subunits*

The data demonstrate that novel GDNF receptor is composed of multiple subunits which cooperate to achieve high affinity binding. The cooperative binding of GDNF to embryonic sympathetic neurons may thus be an indication of a multi-step mechanism of

15   receptor assembly. Because binding assays were performed at 4°C, binding cooperativity is unlikely to have resulted from substantial lateral mobility of transmembrane receptor proteins, suggesting that GDNF binding induces conformational changes on receptor complexes that are partially preformed on the membrane. The nearly identical affinities of the different GDNF receptor subunits obtained by cross linking also support the notion of

20   cooperative binding of GDNF to a partially pre-assembled receptor complex.

The structural similarities between GDNF and members of the TGF-β superfamily suggest that receptors for GDNF might conform to some of the prototypes described for receptors of members of the TGF-β family. Indeed, the pattern of GDNF binding proteins described herein is strongly reminiscent of type I, type II and type III TGF-β

25   receptors.

Despite the overall similarities between GDNF and TGF-β superfamily receptors, no GDNF receptors could be detected in several cell lines known to express various TGF-β and activin receptor subunits, including the mink lung epithelial cell line MvILu.

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In agreement with this observation, no binding of GDNF has been detected in COS cells transfected with different combinations of known type I and type II TGF-β superfamily receptors (Ibanez, C., unpublished; P. ten Dijke, personal communication), including the recently isolated type II receptor for BMPs (Rosenzweig et al., 92 *PNAS USA* 7632,

5   1995) and a novel brain-specific type I receptor (Ryden et al., 21 *Abs. Soc. Neurosci* 1754, 1995). Moreover, no GDNF receptor complexes could be recovered after immunoprecipitation with antipeptide antisera against any of the cloned TGF-β superfamily receptors, indicating that GDNF receptor components are novel proteins.

10   **3. c-RET is a receptor for GDNF**

GDNF receptors were found in a motor neuron-neuroblastoma hybrid cell line, but not in a basal forebrain cell which was also a hybrid with the same neuroblastoma, suggesting that the receptors detected on MN-1 cells represent physiologically relevant motor neuron GDNF receptors. In contrast to raphe nucleus cells, GDNF expression

15   could not be detected in the motor neuron cell line, consistent with a target-derived mode of action for muscle-derived GDNF *in vivo* (Henderson et al., 266 Science 1062, 1994; Trupp et al., *supra*). GDNF binds to and induces tyrosine phosphorylation of the these receptor which were identified as the product of *c-ret*. *c-ret* was also able to mediate GDNF binding and survival/growth responses to GDNF upon transfection into naive

20   fibroblasts. Moreover, dopaminergic neurons of the adult substantia nigra were found to express high levels of *c-ret* mRNA, and c-RET expressing dopaminergic and noradrenergic neurons in the CNS responded to the protective effects of exogenous GDNF *in vivo*. Together, these data indicate that the product of the *c-ret* proto-oncogene encodes a functional receptor for GDNF which may mediate the neurotrophic effects of

25   this factor on dopaminergic, noradrenergic and motor neurons.

The results disclosed herein indicate that the c-RET receptor tyrosine kinase is a signal transducing receptor for GDNF. This finding is surprising, given that all receptors for members of the TGF-β superfamily characterized so far are receptor serine-threonine

kinases (Derynck, R. *Trends Biochem Sci* 19, 548-553 (1994); Attisano et *al.*, *J.Bba-Mol Cell Res,222,* 71-80 (1994)). GDNF is in fact a very divergent member of the TGF-β superfamily, with which it shares primarily the spacing between conserved cysteine residues in the amino acid sequencer. Its ability to interact with a receptor tyrosine

5   kinase indicates a further functional divergence from other members of the TGF-β superfamily. Conversely, these findings could suggest that other TGF-β superfamily members may also utilize receptor tyrosine kinases.

The following results disclosed herein also implicate the *c-ret* proto-oncogene product as a functional receptor for GDNF:

10   i)   GDNF binds to COS cells ectopically expressing the *c-ret* proto-oncogene;

                 ii)   GDNF can be chemically crosslinked to the product of the *c-ret* proto-oncogene ectopically expressed in COS cells or from NB2/a and PC12 cells;

15   iii)   the *c-ret* proto-oncogene product ectopically expressed in COS cells, but also in NB2/a cells, becomes rapidly phosphorylated on tyrosine residues upon GDNF binding;

                 iv)   GDNF promotes biological effects i.e. mitogenic or trophic in cells expressing *c-ret* proto-oncogenic products.

20   GDNF specifically binds to RET-expressing (Figure 19 c, d, h) enteric neurons and the tips of ureteric buds in developing kidney. These tissues were absent or severely reduced in *c-ret*-deficient mice (Schuchardt et al. (1994) *Nature*, 367,380-383; Durbec et al. (1996) *Development*, 122, 349-358). The data disclosed herein further demonstrate GDNF-c-RET complexes from GDNF-responsive and *c-ret*-transfected cells and from

25   embryonic kidney cells. Finally, GDNF time and dose-dependently activates c-RET, and introduction of *c-ret* into GDNF-nonresponsive cells results in GDNF-responsiveness.

4. *Downstream signaling pathways* activated *by GDNF receptor*

Investigation of GDNF signal transducing mechanisms in raphe nucleus and motor neuron cell lines has been conducted. The downstream responses elicited by GDNF in these cells demonstrate that the GDNF binding proteins identified herein

5   represent functional GDNF receptors. The initial biochemical characterization of GDNF signal transduction pathways has identified members of the ERK/MAP kinase family as components of the GDNF signaling mechanism. ERK/MAP kinase activation by phosphorylation is the final step in a cascade of kinases that is set in motion after activation of the Ras pathway by various growth factors, including TGF-β (Yan et al.,

10   269 *J. Biol. Chem.* 13231, 1994; Hartsough and Mulder, 270 *J. Biol. Chem.* 7117, 1995) and nerve growth factor (Thomas et al., 68 *Cell* 1031, 1992; Wood et al., 68 *Cell* 10 II, 1992). More recently, ERK2 has also been shown to form part of the signal transduction pathway activated by several cytokines, such as interferons and interleukins, which are not known to activate Ras (David et al., 269 *Science* 1721, 1995). Whether or not Ras

15   activation is one of the steps in the signaling transduction mechanism of GDNF is an area of further interest.

Interesting differences were found between the patterns of ERK phosphorylation induced by GDNF in raphe nucleus RN33B cells and in motor neuron MN-1 cells. GDNF treatment stimulated very rapid (maximum at 5 min) and transient (undetectable

20   after 60 min) tyrosine phosphorylation of ERKI and ERK2 in RN33B cells, but relatively slower (maximum at 15 min) and more sustained (still detectable after 120 min) phosphorylation of ERK2, but not ERKI, in MN-1 cells. That these differences may have functional significance is suggested by recent observations made in PC12 cells treated with different growth factors. Exposure of PC12 cells to NGF or fibroblast growth

25   factor (FGF) results in neuronal differentiation and in sustained elevation of Ras activity and ERK tyrosine phosphorylation (Qiu and Green, 7 *Neuron* 977, 1991). In contrast, treatment with epidermal growth factor, which stimulates DNA synthesis and proliferation of PC12 cells, results in only transient (<1 hr) activation of Ras and ERKs (Qiu and

Green, 1991). Thus, different time-courses of ERK activation underlie different biological responses in PC12 cells. Taken together, the different patterns of GDNF receptors and GDNF-induced ERK phosphorylation in RN33B and MN-1 cells suggest that different GDNF receptor subunits can cooperate to assemble distinct signaling complexes in

5   different cell types. Whether different GDNF signal transduction pathways underlie the different biological effects of GDNF is an area of further interest.

Upon activation, ERKs translocate to the nucleus where they phosphorylate and thereby regulate the activity of transcription factors which, in turn, control gene expression. Phosphorylation of $p67^{SRF}$ and $p62^{TCF}$ transcription factors recruits them to

10 the serum response element (SRE) in the c-fos gene promoter and stimulates c-fos gene transcription (Gille et al., 358 Nature 414, 1992). Transcription of c-fos is rapidly and transiently induced after various stimuli, including exposure of PC12 cells to NGF (Millbrandt, 83 *PNAS USA* 4789, 1986) and of osteoblastic cells to TGF-β (Machwate et al., 9 *Mol.Endocrin.* 187, 1995).

15 c-fos forms part of the AP-1 transcription factor complex, which is thought to be involved in the regulation of multiple genes, including growth factor, neuropeptide and neurotransmitter synthesizing enzyme genes (Gizang-Ginsberg and Ziff, 4 *Genes Dev.* 477, 1990); Hengerer et al., 87 *PNAS USA* 3899, 1990; Jalava and Mai, 9 *Oncogene* 2369, 1994). The stimulation of c-fos transcription by GDNF indicates a role for AP-1

20 complexes in GDNF-induced gene expression. Thus, c-fos could mediate the increase in the tyrosine hydroxylase (TH) expression observed upon GDNF treatment of central noradrenergic neurons, or the GDNF-induced upregulation of vasoactive intestinal peptide (VIP) and preprotachykinin-A (PPTA) mRNAs in cultured sympathetic neurons from the superior cervical ganglion (Trupp et al., 130 J. Cell Biol. 137, 1995).

25 The effects of GDNF on the survival of differentiated serotonergic raphe nucleus cells indicate that the GDNF receptors identified on these cells are able to elicit relevant biological responses. The fact that cessation of proliferation, differentiation, and GDNF responsiveness were concomitant with increased GDNF receptor expression in these cells,

suggests that GDNF may be a survival factor for developing serotonergic raphe neurons *in vivo*. The data of this patent disclosure suggest a role for ERKs and c-fos in GDNF-mediated neuron survival. This can be directly established using dominant negatives or antisense oligonucleotides.

5   The GDNF receptor subunits and complexes disclosed herein have wide-range applicability. The identification and isolation of GDNF receptor facilitates rational drug design for drugs useful in treating, for example, neuronal disorders, particularly those involving neuronal cell death. As was discussed previously, GDNF has been shown to promote survival of adult substantia nigra neurons *in vivo* following pharmacological

10   treatments and lesions that mimic Parkinsonian syndromes, as well as survival responses in other neuronal cell lines. The drugs can be tested for binding affinity to GDNF receptor, and for their influence on the downstream effect of GDNF disclosed below -- i.e., the phosphorylation of ERK2 and ERKI. As GDNF receptor has also been identified on malignant cell lines, design of drugs for use in cancer therapy is also

15   evident. Further, considering structural similarity with BMP, the development of drugs to be used in treating bone-related diseases, i.e., osteoporosis, and for promoting the healing of fractures is also contemplated.

     Accordingly, isolated receptors according to the present invention can be used, *inter alia*, to screen for compounds or compositions which are analogs and homologs of

20   GDNF. The potential analogs and homologs can be screened initially in competitive binding assays employing either isolated receptor or cell lines expressing the receptor -- i.e., NB2/a cells -- and ^{125}I-labeled GDNF. Methods such as those disclosed in Example 13 can be used. Analog or homolog activity can then be ascertained by further identifying those compounds or compositions which, for example, effect a decrease or increase,

25   respectively, in the tyrosine phosphorylation of the RET proto-oncogene. Methods such as those disclosed in Example 17 can be used. Alternatively, GDNF can be used to screen for and identify other receptors using the above-reference procedures, or variations thereof.

The isolation of GDNF receptor also facilitates the development of antibodies, both polyclonal and monoclonal, against the receptor. These antibodies can be used to purify the receptors themselves, identify other cells expressing GDNF receptor, thereby prompting other therapeutic applications, identify other Type I-interactive receptors, as

5   well as be used as drugs themselves. The antibodies can initially be produced using the ligand/receptor complexes disclosed herein as the immunogens. Antibodies specific for the ligand can be eliminated from the polyclonal serum by absorption with the ligand. Hybridomas for monoclonal production can be selected on the basis of binding of ligand, with the expansion of only those clones which do not bind the ligand

10   uncomplexed with the receptor. The antibodies can be prepared by methods well known to those skilled in the art.

Alternatively, monoclonal and polyclonal antibodies against GDNF receptor and GDNF proteins can be used for the characterization and/or isolation of GDNF receptor molecular clones. Further, anti-GDNF antibodies can potentially be used in a

15   screen for homologs, or in the production of anti-idiotype antibodies which mimic GDNF.

The isolation of GDNF receptor also facilitates the isolation and/or production of nucleic acids for the expression of recombinant GDNF receptor, both *in vitro* and *in vivo,* for diagnostic and therapeutic applications. The term "nucleic acids" as used

20   herein includes, for example, genomic DNA, mRNA, and cDNA. Upon sequencing at least a portion of the GDNF receptor, oligonucleotide primers for isolating genomic DNA for GDNF receptor and receptor mRNA can be developed.

cDNA can be prepared from isolated mRNA. The isolation and production of

25   nucleic acids can be accomplished utilizing methods well known to those skilled in the art using standard molecular biology techniques such as are set forth in Maniatis et al., <u>Molecular Cloning: A Laboratory</u> Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982, incorporated herein by reference. Recombinantly

produced receptors can be used in crystallography studies for rational drug design. Recombinant extracellular domain can be produced and used as a drug in ligand sink applications, e.g., for ligands with antagonistic properties.

The nucleic acids as set forth above can be utilized for gene therapy, using both *in*
5   *vivo* and *ex vivo* techniques. The nucleic acids can also be used to clone other related receptors using, for example, low stringency screens and reversed transcriptase PCR; and to produce cells overexpressing the receptors to screen for other ligands, e.g., by panning, and other materials serving as receptor agonists, antagonists, or partial agonists and antagonists. Alternatively, recombinantly produced receptor itself can be used for
10   the screening assays. Additionally, cells expressing chimeric receptors can be produced using other TGF-β receptor family members to elucidate signal pathways. Intracellular targets of GDNF receptor can be identified using, for example, the yeast two-hybrid system. (Chen, et al., 377 *Nature* 548, 1995, incorporated herein by reference.)

The nucleic acids set forth above can also be used to develop transgenic and/or
15   gene targeted animals. For example, transgenic animals can be developed for testing the effects of the overexpression of GDNF receptor. Procedures can be utilized such as are described in Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1986, and Capecchi, M.R., *Trends Genet*, 5: 70-76, 1989, both incorporated herein by reference.

20   Alternatively, cell lines and transgenic animals unable to express GDNFreceptor can be prepared to ascertain the effects of blocking signaling by GDNF. Procedures such as are set forth in Wurst et al., *Gene Targeting* Vol. 126, edited by A. L. Joyner, IRL Press, Oxford University Press, Oxford, England, pp. 33-61, 1993, incorporated herein by reference, can be utilized.

25   Other applications and modifications are within the spirit and scope of the invention as herein disclosed and will be readily apparent to those skilled in the art.

EXAMPLES

The following Examples are provided for purposes of elucidation and not limitation on the disclosure or claims.

Unless otherwise indicated, binding and biochemical studies were carried out with recombinant rat GDNF produced in Sf21 insect cells using a baculovirus expression system. The protein was produced and purified as previously described (Trupp et al., *supra*, incorporated herein by reference). GDNF protein was quantified after silver staining of SDS/PAGE gels using standard curves obtained with commercial samples of proteins of molecular weight similar to that of GDNF. Purified human TGF-β1 was generously provided by Jun-ichi Koumegawa, Kirin Brewery, Tokyo, Japan. Proteins were labeled with Na-^{125}I by the chloramine-T method to a specific activity of approximately 1×10^8 cpm/μg.

Unless otherwise indicated, binding assays were performed as follows. Cells were incubated with iodinated GDNF in Dulbecco's phosphate buffered saline and 2 mg/ml bovine serum albumin (BSA) on Millipore Hydrophilic Durapore 96-well filtration plates. Following two hours of vigorous shaking at 4°C, the cells were washed twice with ice-cold binding buffer under vacuum. Dried filters were liberated and bound ^{125}I-GDNF quantified in a gamma counter. Non-specific binding was determined by addition of 500-fold excess of cold ligand to the binding mixtures.

For affinity labeling, iodinated proteins were bound to monolayer cultures of primary neurons or cell lines. Prior to binding, dissociated chick sympathetic neurons were cultured for 48 hours in the presence of NGF on polyornithine/laminin coated dishes. Plated cells were incubated with 10 ng/ml ^{125}I-GDNF at 4°C in binding buffer as described above. Ligand/receptor complexes were chemically cross-linked for thirty minutes at room temperature using either disuccinimidyl suberate (DSS) or 1-Ethyl-3(-3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC) (Pierce Chemical, Rockland, IL). Following quenching of the cross-linking reactions, cells were washed twice with 10mM Tris/HCI buffered saline, 2 mM EDTA, 10% glycerol, 1% NP-40, 1%

Triton x-100, 10 μg/ml leupeptin, 10 μg/ml antipain, 50 μg/ml aprotinin, 100 μg/ml

benzamidine hydrochloride, 10 μg/ml pepstatin and 1 mM PMSF(proteinase inhibitors

from Sigma). Cleared lysates were boiled for 5 min in SDS/β-mercaptoethanol buffer,

fractionated by SDS/PAGE on 4-20% gradient electrophoresis gels, and visualized by

5   autoradiography. Molecular weights indicated were obtained by subtracting the weight of

a GDNF ligand monomer, e.g., 25-30 kD, more preferably 23 kD, from the estimated

molecular weights of cross-linked complexes visualized by SDS/PAGE. For affinity

measurements of cross-linked complexes, cells were incubated on plates as above in the

presence of increasing amounts of unlabeled GDNF. These samples were fractionated by

10   gradient SDS/PAGE, gels were then dried and specific bands excised according to

molecular weights determined from autoradiograms, and count in a gamma counter. For

immunoprecipitation of affinity labeled receptor complexes, after binding and cross-

linking with iodinated ligands, cell lysates were cleared and incubated overnight at 4°C

with 5-10 μl of antipeptide rabbit antisera against difference type I, 11 and III TGF-β

15   superfamily receptors (ten Dijke et al., 264 Science 101, 1994) (provided by Peter ten

Dijke, Ludwig Institute for Cancer Research, Uppsala, Sweden). Immunocomplexes were

collected with Protein A-Sepharose (Pharmacia, Sweden), washed in lysis buffer and

boiled for 5 minutes before SDS/PAGE and autoradiography as above.

20

Example 1

GDNF receptors on embryonic sympathetic neurons

GDNF promotes survival of cultured embryonic chicken sympathetic neurons with

similar efficacy and dose response curve as nerve growth factor (NGF) (Trupp et al.,

25   *supra*). Chicken sympathetic neurons, isolated and prepared as previously described

(Trupp et al., *supra*). Saturation binding with iodinated GDNF was carried out on

neurons isolated from embryonic day 10 (E10) chick paravertebral sympathetic ganglia

mechanically dissociated in the presence of trypsin. The preparation was preplated for

two hours on untreated tissue culture plastic in order to enrich in neurons and allow for re expression of receptors. Plots of saturation binding data produced a sigmoidal curve from which a Kd of 400 pM could be approximated (Fig. 1a). In agreement with the sigmoidal behavior of this curve, Scatchard transformation of the data produced an

5   inverted U-shaped curve indicative of cooperative binding (Fig. 1b). The measure of cooperativity of binding can be ascertained from a Hill transformation which produced a positive slope of 1.63 (Fig. 1c), suggesting oligomerization of either ligand or receptor subunits.

In order to identify GDNF binding components on the membrane of sympathetic

10   neurons, chemical cross-linking of ^{125}I-GDNF to these cells was utilized, followed by visualization of the resulting complexes by SDS-PAGE. Gradient gel electrophoresis resolved binding proteins of 70 and 300kD (molecular weights of GDNF receptor subunits reported hereafter were obtained by subtracting the weight of a GDNF ligand monomer, e.g., 23kD, from the estimated molecular weights of cross-linked complexes

15   visualized by SDS/PAGE), resulting in a pattern of bands which resembled that obtained after cross-linking of TGF-β1 to MvILu mink lung epithelial cells (Fig.2). This result suggested that, like TGF-β receptors, GDNF binding proteins may also form an oligomeric receptor system. A large excess of cold ligand displaced iodinated GDNF from the receptor complex indicating the specificity of the labeling.

20

Example 2

GDNF receptors on cell lines

Over thirty cell lines were screened for expression of GDNF receptors using affinity labeling with iodinated GDNF (Table I, *infra*). Except as otherwise noted, all

25   cell lines used in this study are available from and described by the American Type Culture Collection, Rockville, MD. A875 human neuroblastoma was provided by Mart Saarma, University of Helsinki, Finland. CATH.A, a noradrenergic cell line isolated from a tumor in the pons of transgenic mice expressing SV40 T antigen under the

transcriptional control of a tyrosine hydroxylase promoter (Suri et al., 1993), was
generated and provided by Dona Chikaraishi, Tufts University School of Medicine,
Boston, MA. The rat neural stem cell line C17-2 (Snyder et al., 68 Cell 33, 1992) was
generated and provided by Evan Snyder, Harvard medical School, Boston, MA. LAN5

5   human neuroblastoma was provided by Sven Pahlman, Uppsala University, Sweden.
David Hammond, University of Chicago, produced and provided SN6 cells, a hybrid of
mouse basal forebrain cholinergic neurons and the mouse neuroblastoma N18TG2
(Hammond et al., 1986). Human neuroblastoma SY5Y was a provided by David Kaplan,
ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center,

10   Frederick, MD. ST15A rat neural stem cell line was kindly provided by Ron McKay,
National Instituted of Health, MD. The generation and characterization of raphe nucleus
cell lines RN33B and RN46A has been described elsewhere (Whittemore and White,
1993). RN33B and RN46A cells were obtained from Dr. Scott Whittemore of the
University of Miami. The motor neuron hybrid cell line 2FI.10.14 (referred to here as

15   MN-1) has been previously described (Salazar-Grueso et al., 2 *Neuroreport* 505, 1991).

Multiple GDNF receptor subunits were detected in various glial, neuronal and
non-neuronal cells (Table I). A large molecular weight band of 300kD appeared to be
the most prevalent species in several cell lines after cross linking with disuccymidyl
suberate (DSS), and it was the only receptor which appeared to bind ligand in the absence

20   of all other receptors (Table I). A similar pattern was seen in rat C6 glioma, mouse
Sertoli TM4 cells, and in two cell lines derived from embryonic neuronal precursors of
the rat raphe nucleus, which have previously been shown to express multiple neuronal
markers, including glutamate- (RN33B) and serotonin- (RN46A) synthesizing enzymes
(Whittemore and White, 615 *Brain Res.* 27, 1993; White et al., 14 J. Neuro., 1994;

25   Eaton et al., 170 *Dev. Biol.* 169,1995). The consensus pattern in these cells after cross-
linking with DSS consisted of the large molecular weight band of 300kD, and two other
receptor subunits with molecular weights at 50-55kD and 65-70kD, respectively (Fig. 3
and Table I). The 50-55kD component often ran as a doublet or triplet. The smeary

appearance and heterogeneous range of sizes displayed by the large molecular weight
component suggests a post-translational modification, presumably glycosylation, and
appears similar to that previously described for type III betaglycan TGF-β receptors. This
species was somewhat smaller in the cells derived from the raphe nucleus, which could
5   indicate either a distinct core protein or difference levels of glycosylation.

GDNF receptors could not be detected in pheochromocytoma PC12 cells under the
present assay conditions of 4°C, even after NGF-induced differentiation into a
sympathetic neuron-like phenotype (Table I, and data not shown). No or very low GDNF
receptor expression could be seen in various neuroblastomas, and in two pluripotent
10   neuronal stem cells (Table I).

TABLE I

CELL LINE	DESCRIPTION	55kD	70kD	135kD	155kD	300kD
5 A875	human melanoma	-	-	-	-	+
Balb.SFME	mouse embryonic cell	-	-	-	-	-
CATH.a	rat pons noradrenergic	-	-	-	-	-
Mv1Lu	mink lung epithelia cell	-	-	-	-	-
COS-7	monkey kidney fibroblast	-	-	-	-	-
10 C2-C12	mouse myoblast	-	-	-	-	-
C6	rat glioma	+	+	+	-	+
C17-2	rat CNS stem cell	-	-	-	-	-
FR-3T3	rat fibroblast	-	-	-	-	-
HELA	human cervical carcinoma	-	-	-	-	-
15 LAN5	human neuroblastoma	-	-	-	-	+
L6	rat myoblast	-	-	+	-	+
MN-1	mouse motor neuron	-	+	-	+	+
NB41A3	TH+ mouse neuroblastoma	-	-	-	-	-
NRK-49F	rat kidney fibroblast	-	-	-	-	-
20 PC12	rat pheochromocytoma	-	-	-	-	-
P19	mouse embryo carcinoma	-	-	-	-	-
RN33B	rat raphe nucleus (glutamat)	+	+	+	-	+
RN46A	rat raphe nucleus (seroton)	+	+	+	-	+
SK-N-MC	human neuroepithelioma	-	-	-	-	-
25 SK-N-SH	DRH+ mouse neuroblastoma	-	+	-	-	+
SN6	mouse basal forebrain (cholin)	-	-	-	-	-
ST15A	rat CNS stem cell	-	-	-	-	+
SW1353	human chondrosarcoma	-	-	-	-	-
SY5Y	human neuroblastoma	-	-	-	-	-
30 TM3	mouse Leydig cell	-	-	-	-	-
TM4	mouse Sertoli cell	+	+	+	-	+
U138MG	human glioblastoma	-	-	-	-	-

Presence (+) or absence (-) of specific GDNF receptor complexes in the designated cell
35 line.

Affinity labeling using the cross-linker ethyl-dimethyl-aminopropyl carbodiimide

(EDAC) revealed the presence of an additional GDNF receptor component of 120-135kD

(Fig. 3), only seen after very long exposure of gels in DSS cross-linked complexes. Like

DSS, EDAC also cross-linked GDNF to receptors of 50-55kD and 65-70kD; the high

molecular weight subunit of 300kD was, however, not as efficiently cross-linked by

5   EDAC (Fig.3).

The raphe nucleus cell lines are only conditionally immortalized and do not show

signs of transformation. At the non-permissive temperature and in defined medium, they

stop proliferating and differentiate into postmitotic neurons (Whittemore and White,

1993). GDNF binding was greatly increased in RN33B and RN46A cells following

10   differentiation (not shown). The overall pattern and the relative amounts of GDNF

receptor components did not change after differentiation.

Analysis of GDNF binding proteins on the rat myoblast cell line L6 revealed a

different pattern of receptor subunits marked by the apparent absence of 50-55kD and 65-

70kD receptors. Only the high molecular weight component of 200-400kD could be

15   seen after cross-linking with DSS (Fig. 3). Cross linking with EDAC, however, readily

labeled the 120-135kD subunit previously seen in C6, TM4 and raphe nucleus cell lines

(Fig. 3). As in these other cell lines, this component also run as doublet in L6 myoblasts.

A distinct receptor complex was found on an embryonic mouse spinal cord motor

neuron hybrid cell (Fig. 3). This line was obtained by fusion of E14 mouse spinal cord

20   motor neurons and the N18TG2 mouse neuroblastoma, followed by selection of clones

expressing high levels of choline acetyltransferase activity (Salazar-Grueso et al., 2

Neuroreport 505, 1991). Importantly, SN6, a hybrid cell line of embryonic mouse basal

forebrain cholinergic neurons and the same N18TG2 neuroblastoma (Hammond et al.,

234 Science 1237, 1986), showed no GDNF receptors (Table I), indicating that the

25   GDNF binding proteins seen on the motor neuron cell (hereafter referred to as MN-1) are

likely to represent GDNF receptor components present in spinal motor neurons. As with

the L6 myoblasts, the predominant receptor in MN-1 cells was preferentially cross-linked

with EDAC, although in these cells it was a larger protein of 155kD (Fig. 3). This was

subsequently identified to be a c-RET receptor (see Example 9 below).   MN-1 cells also expressed 65-70kD binding proteins and low amounts of the 300kD receptor (Fig. 3 and Table 1).

In order to dissect the individual constituent affinities of GDNF receptor subunits,
5   displacement binding assays were performed, followed by cross-linking and SDS-PAGE. Receptor-ligand complexes were visualized by autoradiography, cut out from the gel and counted in a gamma counter. The resulting displacement curves indicated a Kd of approximately 0.2 nM for all components on RN33B and MN-1 cells (Fig. 4 a-c). These data at present do not clearly establish whether all GDNF receptor subunits display
10   similar binding affinities or, whether they are all required to assemble a high affinity receptor complex.

Example 3

Biochemical characterization of GDNF receptors

15   The overall similarity in the pattern of receptors between GDNF and TGF-β prompted an examination of whether any of the previously identified receptors for TGF-β superfamily members was part of the GDNF receptor complex. Cross-linked ^{125}I-GDNF-receptor complexes from differentiated RN33B cells were subjected to immunoprecipitation with different anti-peptide antisera specific for all cloned TGF-β
20   superfamily receptors, including type I receptors (ALK-1 to ALK-6), type II receptors TBRII, ActRII and BMPRII, the type III receptors betaglycan, and endoglin. In a parallel control experiment, ^{125}I-TGF-β1 was cross-linked to type I, type II and type III receptors on the mink lung epithelial cell line MvILu followed by immunoprecipitation with antisera against TBRI (ALK-5), TBRII and betaglycan, respectively. Although type
25   I, type II and type III TGF-β receptors were recovered in the control experiment, none of the GDNF receptor components in differentiated RN33B cells could be immunoprecipitated by any of the tested antisera (not shown). These data confirmed that the GDNF receptor subunits expressed on these cells are novel proteins.

Example 4

Endogenous GDNF expression in cell lines expressing GDNF receptors

Traditional models for the action of neurotrophic factors have described them as target-derived polypeptides that promote survival and differentiation of specific neuronal

5   subpopulations. More recently, it has become evident that neurotrophic factors may also have paracrine and even autocrine modes of action (Ernfors and Persson, 3 *Eur. J. Neurosci.* 953, 1991; Acheson et al., 374 *Nature* 450, 1995). Expression of GDNF mRNA in cell lines expressing GDNF receptors was examined. Cells were homogenized in guanidine isothiocyanate (GITC) and β-mercaptoethanol. RNA extraction and GDNF

10   RNAse protection assay were as previously described (Trupp et al., *supra*).

Unexpectedly, all cell lines, with the exception of the motor neuron line MN-1, expressed substantial levels of GDNF mRNA as assayed by RNAse protection analysis (Fig. 5). The highest GDNF mRNA expression was found in cells from raphe nucleus, which showed up to 5-fold higher expression than postnatal day 1 (P1) kidney, one of the

15   richest sources of GDNF mRNA in the developing rat (Trupp et al., *supra*). Interestingly, upon differentiation of RN33B cells, GDNF mRNA expression decreased to about 30% of the level in undifferentiated cells (Figure 5). GDNF treatment of differentiated RN33B cells did not alter the expression of GDNF mRNA (Fig. 5) or GDNF receptors (not shown).

20   Expression of *c-ret* mRNA was investigated in RN33B, L6, and MN-1 cells, using the RNAse protection assay. Ten micrograms of total RNA from the cell lines indicated was analyzed using a riboprobe complementary to 400 nucleotides of coding sequence from the kinase domain of the mouse *c-ret* mRNA. Although high expression was seen in MN-1 cells, no *c-ret* mRNA was detected in either the RN33B or L6 cells (Fig. 6).

25   These results indicate that a signaling receptor for GDNF other than c-RET must be present in these cells.

Example 5

Activation of the ERK signal transduction pathway in GDNF responsive cell lines

Whether the GDNF binding proteins characterized in cell lines were able to form ligand-dependent signaling complexes was also investigated. Cell monolayers in 10 cm plates were incubated at 37°C in the presence of 50 ng/ml GDNF for the indicated time periods and immediately lysed with 1 ml of ice cold lysis buffer (as above) with the addition of 1 mM sodium othovanadate. Whole cell lysates were fractionated by SDS-PAGE (10% polyacrylamide) and blotted to nitrocellulose filters. Western blots were probed with an anti-phosphotyrosine antiserum (UBI, Lake Placed, NY), followed by horseradish peroxidase-conjugated goat anti-mouse IgG and developed with the ECL Western Detection System (Amersham, UK). For reprobing, blots were first stripped by a 30 minute incubation at 50°C in 62.5 mM Tris-HCI pH6.7, 100 mM β-mercaptoethanol, 2% sodium dodecyl sulphate. After removal of antibodies, blots were probed with a rabbit polyclonal antisera raised against recombinant rat ERK2 (a gift of Teri Boulton, Regeneron Pharmaceuticals Inc., Tarrytown, NY) which recognizes both ERKI and ERK2, and developed as above using a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody.

Because of their distinct patterns of GDNF receptor subunits, intracellular signaling responses were initially characterized in the raphe nucleus cell line RN33B and in the motor neuron cell line MN-1. Changes in the pattern of tyrosine-phosphorylated proteins elicited by GDNF treatment of RN33B or MN-1 cells were investigated. Tyrosine phophorylation is a universal mechanism of regulation of intracellular signaling proteins that is stimulated by numerous cytokines and growth factors. RN33B and MN-1 monolayers were exposed to a saturating concentration of GDNF (5 ng/ml) for different periods of tine, and total cell lysates were analysed for tyrosine phosphorylation by SDS/PAGE and Western blotting with an anti-phosphotyrosine monoclonal antibody. Two proteins with mobilities corresponding to 42kD and 44kD, respectively, were phosphorylated on tyrosine within 5 minutes of GDNF treatment of RN33B cells (Fig.

7A). A similar result was obtained in differentiated RN33B cells (not shown) of exposure to GDNF.

Based on comparison of their size with descriptions of growth factor-induced protein tyrosine phosphorylation elsewhere (Qiu and Green, 9 Neuron 705, 1992), the

5   42kD and 44kD species would appear to be, respectively, p42 ,k2 and p44 ,kI , two protein serine-threonine kinases members of the extracellular signal-regulated kinase (ERK, also termed microtubule-associated protein kinase) family (Boulton et al., 65 Cell 663, 1991). To confirm the identity of these proteins as ERK2 and ERKI, respectively, protein blots which had been reacted with the anti-phosphotyrosine antibody were stripped

10   and reprobed with a rabbit polyclonal antibody raised against recombinant ERK2 that recognizes both ERKI and ERK2 in protein blots. Comparison of autoradiograms of blots probed with the anti-phosphotyrosine antibody and the anti-ERK2 antibody identified the p42 and p44 proteins as ERK2 and ERKI, respectively (Fig. 7a). Although GDNF treatment of MN-1 cells appeared to only stimulate phosphorylation of ERK2, both ERKI

15   and ERK2 were present in MN-1 cell lysates (Fig. 7b). Thus, GDNF treatment stimulated very rapid and transient tyrosine phosphorylation of ERKI and ERK2 in RN33B cells, but relatively slower and more sustained phosphorylation of ERK2 and MN-1 cells.

Activation of the ERK pathway has previously been shown to induce rapid and

20   transient increase in transcription of immediate early genes, including the c-fos proto-oncogene (Gille et al., 358 Nature 414, 1992). Accordingly, the ability of GDNF to induce c-fos mRNA in differentiated raphe nucleus RN33B cells and in motor neuron MN-1 cells was investigated. For analysis of c-fos mRNA expression in cell lines, culture medium was changed 90 minutes prior to addition of 100 ng/ml GDNF to cell

25   monolayers. At the indicated time intervals, media was removed, cells solubilized with guanidine isothiocyanate and β-mercaptoethanol and RNA extracted as previously described (Trupp et al., supra). Twenty micrograms of total RNA was fractionated on 1% agarose gels containing 0.7% formaldehyde and transferred to Hybond-C membranes

(Amersham, UK). Northern blots were hybridized with an a-^{32}P-dCTP labeled rat c-fos gene fragment (Curran et al., 2 *Oncogene* 79, 1987), washed at high stringency and visualized by autoradiography on x-ray films.

5   Cell monolayers were exposed to saturating concentrations of GDNF for different periods of time and levels of c-fos mRNA were subsequently analyzed in Northern blots of total RNA (Fig. 8). This analysis revealed transient upregulation of c-fos mRNA 15 minutes after exposure of RN33B cells to GDNF, returning to basal levels 45 minutes after treatment (Fig. 8a). c-fos mRNA was also upregulated in MN-1 cells but not until 30 minutes of GDNF treatment (Fig. 8b). Elevated c-fos mRNA levels persisted for

10   about an hour and returned to basal levels 120 minutes after the initiation of treatment (Fig. 8b). Thus, like tyrosine phosphorylation of ERKS, c-fos mRNA upregulation induced by GDNF treatment was very rapid and transient in RN33B cells, but somewhat slower in MN-1 cells.

15

Example 6

Survival responses promoted by GDNF in differentiated raphe nucleus cells

      Advantage was taken of the conditional nature of the immortalization of the

20   raphe nucleus serotonergic cell line RN46A by examining whether GDNF may be a survival factor for differentiated raphe nucleus neurons. Survival assays were performed as previously described (Eaton et al., 1995, *supra*). Briefly, 10^5 RN cells were seeded to collagen/fibronectin coated 8-well glass slides and incubated at 33°C (growth permissive temperature) until 75-90% confluent. The slides were then shifted to 39°

25   (non-permissive temperature) and serum containing medium was replaced by B16 defined medium (Brewer and Cotman, 494 *Brain Res.* 65,1989) containing 1% BSA, 1 μg/ml transferrin, 5 μg/ml insulin, 100 mM putrescine, and nM progesterone plus or minus 0-50 ng/ml rhGDNF (Promega, Madison, WI). Media and GDNF were replaced

every two days for 8 days after which the cells were fixed in 4%

paraformaldehyde/2% glutaraldehyde, rinsed and coated with a glycerol mounting

medium containing 1 mM bisbenzamide (Hoechst dye 33342) to stain viable nuclei.

Fields of cells were magnified to 40x on a Zeiss Axiophot microscope, examined for

5   fluorescent nuclei (at 355 nM excitation, 465 nM emission), the images video captured,

and the cells counted with Imade I™ software. For each condition, 10 fields of cells were

counted from each of 3 independent experiments.

RN46A cells were cultured at the non-permissive temperature in defined medium

in the presence of increasing concentrations of GDNF. Nine days after plating, surviving

10   cells were counted and compared with cultures established in the absence of GDNF. A

3-fold increase in the number of surviving cells was observed in cultures grown in the

presence of GDNF (Fig. 9). The effect of GDNF on the survival of differentiated

RN46A cells was dose dependent, with an EC50 at 5ng/ml.

15

Example 7
Generation, cloning and characterization of anti-GDNF monoclonal antibodies
Immunisation

20   Five young female mice were immunised with 35 ug of insect cell-derived

recombinant GDNF emulsified with complete Freund's adjuvant (FA). Second and third

immunizations were performed 2 and 4 weeks after the first one in incomplete FA. All

the injections were given intraperitoneally (i.p.). Two weeks after the last Immunisation,

antibody titer in serum was checked by ELISA and Western Blot analysis using standard

25   methods. The mouse with the highest titer (more than 1:2000) was boosted i.p. with 3 μg

of GDNF in incomplete FA 3 days before the cell fusion.

Cell fusion

Cell fusion was done according to the method of Kohler and Milstein (1975),

incorporated herein by reference, with some modifications.

a) Day before fusion:

Viable cells from the Sp2/0 murine cell line were adjusted to $2x10^5$ cells / ml with complete DMEM (10% fetal calf serum, 1% L-glutamine, 100 U/ml penicillin and

5   100 ug/ streptomycin sulphate).

Cells from a non-immunised mouse were obtained from the peritoneal cavity by injection of 0.34M sucrose solution. The cells were resuspended in complete DMEM containing: hypoxanthine $100\mu M$; aminopterin 0.4M; and thymidine 16 μM, (HAT medium), to $1x10^5$ cells /ml. 100 μl of the cell suspension was added to the 60 inner

10   wells of 96 well plates and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air. These cells were the source of growth factors.

b) Fusion

Spleen cells from the mouse exhibiting the highest serum titer (see above) were homogenized in 10 ml DMEM removing surface fat and other adhering tissue in a sterile

15   hood.

$4.2x10^7$ Sp2/0 cells were fused with 8.4 x 10^7 spleen cells in a solution of melted PEG (3000-3700, Hybri-Max, Sigma). The cells were then grown in HAT medium at 37° C in an atmosphere of 5% CO_2 in air. After one week of culture, the wells were inspected. When hybrids cells covered 10 to 50% of the surface area of the well, the

20   culture supernatants were assayed for antibody by ELISA.

For the ELISA, wells of microplates (Costar, EIA/RIA plate high binding) were coated with 100 ul of 2ug/ ml of GDNF diluted in carbonate/ bicarbonate buffer, pH 9.6. After an overnight incubation at 4 °C, the wells were washed with 0.05M phosphate buffered saline, pH 7.2, containing 0.05% Tween (PBS-T). Nonspecific binding

25   was blocked with PBS-T containing 3% non-fatty milk and 1% goat normal serum. Supernatant samples were incubated 4 hours at room temperature. Peroxidase goat anti-mouse antibody was used and the substrate was o-phenylenediamine dihydrochloride (OPD). Plates were read at 492 nm in an ELISA reader. Negative controls included

completed medium and normal mouse serum.

The hybrids were grown in HAT medium up to two weeks after fusion. Cells were subsequently grown in HT medium until the completion of two cloning procedures, using the limiting dilution method. After each step (when cells reached 10 to

5   50% confluence), assays for specific antibody in supernatants were done by ELISA. Upon recloning, 5 positive hybridoma clones were chosen and the cells were maintained in complete DMEM for 30 days.

Isotyping of monoclonal antibodies

The class and subclass of the monoclonal antibodies were determined by ELISA

10   using a DAKO panel for isotyping of mouse monoclonal antibodies. All five 5 monoclonal antibodies were characterized as IgG_1.

Purification of monoclonal antibodies

Monoclonal antibodies from culture supernatants were purified by Protein G Sepharose fast flow (Pharmacia, Biotech) according to manufacturer's instructions.

15   Culture supernatants were concentrated and filtered through a 0.45 μm membrane (Schleicheer and Schull, Germany) and then pumped overnight through the column previously equilibrated with 20mM sodium phosphate, pH 7.0. Ig was eluted with 0.05M glycine buffer.

20   **Example 8**

A motor neuron cell line showing biological and biochemical responses to GDNF

MN-1 cell monolayers were exposed to increasing concentrations of GDNF in serum-free medium and assayed 3 days later for cell survival and growth by measurement

25   of acid phosphatase activity (Clontech). GDNF was produced and purified from baculovirus infected insect cells as previously described (Trupp et al.,*supra*). GDNF treatment of serum deprived-MN-1 monolayers increased cell number in a dose-dependent manner (Fig. 10a). The biological response of MN-1 cells correlated with

biochemical and transcriptional responses to GDNF treatment. MN-1 cell monolayers were exposed to 50 ng/ml GDNF for increasing periods of time, cell lysates were fractionated by SDS/PAGE and Western blots probed with an anti-phosphotyrosine antibody (UBI).

5   Several proteins were seen to have increased tyrosine phosphorylation after GDNF treatment of MN1 cells, including a protein with an electrophoretic mobility of 42K (Fig. 10b). Based on comparison of its size with descriptions of growth factor-induced protein tyrosine phosphorylation elsewhere (Boulton, T.G., *et al.* *Cell* 65, 663-75 (1991), the 42K species would appear to be p42^{erk2}, a serine-threonine kinase member of the

10   extracellular signal-regulated kinase (ERK) family. The identity of this protein as ERK2 was confirmed after immunoprecipitation with an antiERK2 polyclonal antiserum followed by analysis of tyrosine phosphorylation (Fig. 10c). Lysates of GDNF-stimulated MN-1 cells were immunoprecipitated with an anti-ERK2 antiserum (Santa Cruz) that also recognizes ERKI followed by antiphosphotyrosine Western blotting. This analysis further

15   revealed that another member of the ERK family, p44erk1, was also phosphorylated on tyrosine after GDNF treatment of MN-1 cells (Fig. 10c). Activation of the ERK pathway has previously been shown to induce a rapid and transient increase in the transcription of immediate early genes, including the *c-fos* proto-oncogene (Gille et *al.*, *Nature* 358, 414-7 (1992).

20

Example 9

The product of the *c-ret* proto-oncogene as a signal transducing receptor for GDNF

       GDNF receptor complexes from MN-1 cells could be recovered by immunoprecipitation with anti-GDNF antibodies or by binding to lectin-Sepharose beads

25   (Fig. 11a). Unexpectedly, the 180kD receptor complex (i.e., c-RET; 180kD - 23kD = 157kD, which is approximately equal to the 155kD receptor identified as c-RET -- see Example 2, *infra*) could also be recovered by immunoprecipitation with anti-phosphotyrosine antibodies (Fig. 11a), indicating that the GDNF binding protein in this

complex could be a receptor tyrosine kinase.

The product of the *c-ret* proto-oncogene is highly expressed in primary motor neurons (Pachnis et al., *supra,* and Tsuzuki, T., *et al. Oncogene 10,* 191-8 (1995) and is of similar molecular weight as the major GDNF receptor component detected in MN-1

5   cells (Takahashi, M., *et al. Oncogene* 3, 571-578 (1988). We tested whether this species represented a C-RET-GDNF cross-linked complex by immunoprecipitation with anti-c-RET antibodies.

^{125}I-GDNF was cross-linked to MN-1 cells using EDAC and receptor complexes were precipitated with antibodies against GDNF (Trupp et al., *supra),* lectin Sepharose

10   beads (Formica), anti-phosphotyrosine antibodies (UBI), anti-c-RET antibodies (Santa Cruz) and control antibodies from non-immune rabbits. An antipeptide c-RET rabbit antiserum readily immunoprecipitated the major 180kD ligand-receptor complex in MN-1 cells (Fig. 11a), while a number of unrelated monoclonal and polyclonal antibodies used as controls failed to immunoprecipitate this complex (Fig. 11 a and data not shown).

15   Because the product of the *c-ret* gene is a receptor tyrosine kinase, we investigated whether GDNF could stimulate tyrosine phosphorylation of the c-RET protein in MN-1 cells. MN-1 cell monolayers were exposed to GDNF at different concentrations or for different periods of time and cell lysates were immunoprecipitated with anti-c-RET antibodies and analyzed by SDS/PAGE and Western blotting with antiphosphotyrosine

20   antibodies as disclosed above. GDNF treatment stimulated rapid c-RET tyrosine phosphorylation in MN-1 cells (Fig. 11b). Maximal phosphorylation was reached 5 minutes after GDNF treatment and lasted for at least 60 minutes. A dose-response analysis of GDNF induced c-RET phosphorylation in MN-1 cells showed maximal phosphorylation at 30 ng/ml of GDNF (Fig. 11b), which is similar to the response of both

25   serum deprived MN-1 cells (Fig. 10a) and embryonic sympathetic neurons (Trupp et al., *supra)* to GDNF. Taken together, these data indicate that the c-RET receptor may be an important component in the signal transduction mechanism of GDNF.

Example 10

c-ret **transfection reconstitutes GDNF binding and biological activities to GDNF**

Experiments were conducted to determine whether expression of the *c-ret* gene product could be sufficient to allow binding of GDNF to cells lacking GDNF receptors.

5   To this end, GDNF binding and cross-linking experiments were performed in naive 3T3 fibroblasts, and 3T3 cells stably transfected with either a wild type *c-ret* or an oncogenic form of this gene found in MEN2a patients (Mulligan et al., *Nature*, 363:458-460, 1993). For *c-ret* expression in transfected cells, human wild type *c-ret* and MEN2a-ret cDNAs were subcloned in pcDNA3 (Invitrogen). Cold GDNF was used at 50x molar excess. For

10   survival/growth assays, cells were cultured for 6 days in serum-free medium supplemented with the indicated concentrations of GDNF; medium and GDNF were replaced every two days. Cell number was quantified by measurementof acid phosphatase activity (Clontech).

After immunoprecipitation with c-RET antibodies, GDNF-labeled receptor

15   complexes of approximately 180K were detected in both MEN2a-ret and *c-ret* transfected 3T3 fibroblasts, but not in untransfected cells (Fig. 12a). The labeling could be displaced by excess cold GDNF, indicating that it represented specific GDNF binding (Fig. 12a).

Experiments were also conducted to determine whether *c-ret* could mediate a biological response to GDNF upon transfection in non-responsive cells. Survival and

20   growth responses to GDNF were investigated in untransfected and *c-ret* transfected 3T3 fibroblasts cultured in serum-free medium. GDNF elicited a dose-dependent increase in cell number in *c-ret* transfected, but not in untransfected, 3T3 cells (Fig.12b) which was comparable to the one previously observed in serum-deprived MN-1cells. Since naive 3T3 cells did not express any appreciable amount of GDNF receptors prior to transfection

25   (see Fig. 12a), it was concluded that *c-ret* expression was sufficient for mediating a biological response to GDNF in these cells.

Example 11

c-ret expression in adult brain and dopaminergic neurons of the substantia nigra

Experiments were conducted to determine whether the c-ret product may mediate the neurotrophic effects of GDNF in the brain by examining the expression of c-ret in

5   different regions of the rat central nervous system. A rat c-ret riboprobe was generated using as template a cDNA fragment obtained by PCR with primers based on sequences U22513 and U22514 (Genbank accession numbers). High levels of c-ret mRNA were found in MN-1 cells and in rat spinal cord (data not shown). High c-ret mRNA expression was also found in the adult pons, medulla, locus coeruleus and hypothalamus

10   (Fig. 13a), as well as in thalamus and cerebellum (data not shown). c-ret mRNA was expressed at barely detectable levels in striatum, hippocampus and cerebral cortex (Fig. 13a). In the ventral mesencephalon, containing the cell bodies of GDNF-responsive dopaminergic neurons, c-ret mRNA levels increased progressively during post-natal development (Fig. 13b). A peak of expression was detected between post natal day 6

15   (P6) and P8, at which time axons of dopaminergic neurons of the substantia nigra begin innervation of the striatum, and coincident with an increase in GDNF mRNA expression in this target region (Fig. 13b). For mRNA quantification, a glyceraldehyde-3-P dehydrogenase (GAPDH) riboprobe was included in the RPA, and values of relative mRNA expression, obtained after densitometric scanning of gel autoradiograms, were

20   normalised using the GAPDH signal of each RNA sample. RPA for GDNF mRNA has been previously described (Trupp et al., *supra).

In situ hybridisation and immunohistochemistry were performed as previously described (Arenas, E. & Persson, H. *Nature* 367, 368-371 (1994); Neveu, I & Arenas, E. *J. Cell Biol.* in press (1996). c-RET protein was detected using a hamster monoclonal

25   anti-mouse c-RET antibody which also recognises rat c-RET (Lo, *supra*) followed by fluorescein-conjugated rabbit anti-hamster secondary antibodies (Southern Biotechnologies). *In situ* hybridization on sections through the adult substantia nigra revealed strong labelling over neurons throughout this structure (Figs. 14 a-b). In

addition, cells positive for c-RET-like immunoreactivity (c-RET-LI) were found

throughout the adult substantia nigra, with strong labelling over cell bodies (Fig. 14c).

In order to establish that *c-ret* expression in the adult substantia nigra was

confined to dopaminergic neurons, these cells were selectively lesioned with a unilateral

5   injection of 6-hydroxydopamine (6-OHDA); the cells were then analyzed for *c-ret* mRNA

expression by *in situ* hybridisation. Lesions of dopaminergic neurons of the substantia

nigra were performed by stereotaxic injections of 8 pig 6-OHDA in the medial forebrain

bundle at the following coordinates: 1.6 mm caudal to bregma, 1.3 mm lateral to

midline, and 8.4 mm under the dural surface with the incisor bar 5 mm over the

10   interaural line. Animals were pretreated with 25 mg/kg desipramine (i.p.) 30 minutes

prior to 6-OHDA injection. 0.75×10^6 GDNF-expressing fibroblast cells in 3 μl of

medium were injected supranigrally at the following coordinates: 3,1 mm from interaural

line, 2 mm lateral to midline, and 7 mm under the dural surface, with the incisor bar at -

3.3 mm. Lesion and grafting in the locus coeruleus were as previously described

15   (Arenas et al., *Neuron* 15, 1465-1473 (1995). The generation and characterisation of

GDNF expressing fibroblasts have been described previously (Arenas et al., supra).

Five hours after the lesion, no difference could be seen between ipsi and

contralateral sides in *c-ret* mRNA expression (Fig. 14d). However, a marked reduction

in *c-ret* mRNA expression was seen in the lesioned substantia nigra already one day

20   after 6-OHDA treatment, and was nearly absent 5 days after the lesion (Fig. 14d). *c-*

ret mRNA expression in the side contralateral to the lesion was, however, not affected

(Fig. 14d). This result indicated that in the adult substantia nigra, c-ret mRNA expression

was confined to dopaminergic neurons.

25   **Example 12**

GDNF rescues c-RET-positive dopaminergic and noradrenergic neurons

Experiments were conducted to determine whether c-RET expressing neurons of

the adult substantia nigra and locus coeruleus responded to GDNF. For this, nigral

dopaminergic neurons lesioned with 6-OHDA, and were then examined to determine whether grafts of GDNF expressing fibroblasts induced responses on c-RET immunoreactive neurons. In lesioned animals that received a graft of control fibroblasts, no c-RET-LI could be detected, indicating a depletion of c-ret-expressing cells by

5   selective lesion of dopaminergic neurons in the adult substantia nigra (Fig. 14e). However, c-RET-LI could be rescued by the GDNF-expressing graft, where c-RET immunopositive fibers could be seen surrounding and penetrating the graft (Fig. 14f). Similar results were obtained in the locus coeruleus, where lesion with 6-OHDA depleted c-RET-immunoreactive cell bodies (Fig. 14g), which could be rescued by exogenous

10   administration of GDNF (Fig. 14h). In both brain regions, the rescue of c-RET-LI positive cells and sprouting in the animals grafted with GDNF-expressing fibroblasts paralleled that of tyrosine hydroxylase immunoreactivity (data not shown), demonstrating that c-RET-expressing adult dopaminergic and noradrenergic neurons respond to GDNF.

15   **Example 13**

Identification of GDNF c-RET receptors

PC12 cells and NB2/a cells were washed three times with serum free RPMI-1640 or DMEM, respectively, plated on noncoated (NB2/a cells) or collagen-coated (PC12 cells) dishes (5000-6000 cells per dish) in the presence or absence of 50 ng/ml of GDNF

20   (Peprotech EC Ltd.) and the number of cells was microscopically counted after 48 hours. PC12 and NB2/a cells were harvested (100,000 cells, five parallels), incubated with 10 ng/ml human ^{125}I-GDNF (iodinated by Chloramine T method, 100 μCi/μg) in the presence or absence of 50-fold unlabeled GDNF for 120-150 min on ice, the unbound factor was removed by centrifugation through 30% sucrose cushion, and the cell-

25   associated radioactivity counted on 1271 RIAGAMMA counter (LKB Wallac).

Recombinant human GDNF promoted survival of about 20% of serum deprived rat pheochromocytoma PC12 cells at concentration of 50 ng/ml (Fig. 15a). Serum-deprived PC12 cells are also maintained by nerve growth factor (NGF). Upon treatment

with (NGF), PCI2 cells also stop dividing and differentiate into sympathetic neuron-like cells with long neurites. Thus, GDNF is a survival-promoting factor for PCI2 cells, although less potent than NGF, but it does not induce differentiation of PC12 cells at the concentrations studied, presumably because of the differences in signal transduction of

5   NGFactivated trkA receptors and GDNF receptors.

Human neuroblastoma NB2/a cells were plated in serum-free medium in the presence or absence of 50 ng/ml of GDNF and the number of cells was counted after 48 hr of culture. GDNF significantly increased the number of NB2/a cells (Fig. 15b). Monkey COS cells, human SY5Y cells and mouse NIH 3T3 cells showed neither

10   mitogenic nor survival response to GDNF (data not shown). Thus, GDNF exerts biological effects on rat PC12 cells and human NB2/a cells, indicating that both cell lines express functional GDNF receptors.

To determine, whether GDNF binds to the responsive cells, PC12 cells and NB2/a cells were incubated with ^{125}I-labeled human GDNF at 40°C as indicated in the legend to

15   Fig. 15. As shown in Fig. 15c, both PC 12 and NB2/a cell lines bind GDNF 30 efficiently. More importantly, the binding of ^{125}I-labeled GDNF could be competed with a 50-fold excess of unlabeled GDNF (Fig. 15b). Thus, the binding of GDNF to the receptors on PCI2 and NB2/a cells appears to be specific.

20   **Example 14**

Identification of GDNF c-RET binding components

PC12 cells, SY5Y neuroblastoma cells and NB2/a cells where chemically cross-linked to ^{125}I-GDNF with EDC. 3-5 x 10^6 cells or mechanically dissociated cells from 2 E20 rat kidneys were incubated with 10 ng/ml of ^{125}I-GDNF for I hour on ice and cross

25   linked with 30 mM EDAC (Pierce) for 30 minutes on ice. Detergent lysates were immunoprecipitated, the precipitates collected by Protein A-Sepharose, separated on 7% SDS-PAGE, and visualized by Phosphorimager SI (Molecular Dynamics).

The resulting complexes were immunoprecipitated with rabbit antibodies to

GDNF, analyzed by SDS-PAGE and visualized by autoradiography. Embryonic kidney cells were also studied as the source of putative GDNF receptor (Suvanto, P. et. al., *Eur. J. Neurosci.,* 8, 101-107 (1996); Sainio, K. et. al., *Nature,* (1996) submitted). Cross-linked complexes of 170 and 190 kD were obtained from the extracts of PC12 cells,

5   SY5Y cells and NB2/a cells and a 190 kD complex from embryonic kidney extracts. (Fig. 16).

The molecular weights of the cross linked proteins minus GDNF of approximately 25-30 kD, substantially, if not exactly, correspond to the molecular weights of c-RET protooncogene, an orphan receptor tyrosine kinase (Takahashi, M., Ritz, J. & Cooper,

10   G. *M. Cell,* 42, 581-588, 1985; Takahashi, M. et.al., *Oncogene,* 3, 571-578 (1988)) (140 kD and 160 kD, representing differently glycosylated forms of c-RET., Tsuzuki, T., Takahashi, M., Asai, N., Iwashita, T., Matsuyama, M. & Asai, J. *Oncogene, 10,* 191-198 (1995).

15   **Example 15**

Affinity Cross Linking of GDNF to c-RET

The cross linked complexes were immunoprecipitated from the NB2/a cells with the cocktail of antibodies recognizing extracellular and intracellular part of the c-RET receptor. As shown in Fig. 17a (lane 1), the complexes of 170 kD and 190 kD were

20   precipitated by anti-c-RET antibodies, which thus correspond to cross linked GDNFc-RET complexes. Binding of ^{125}I-GDNF to c-RET proteins was completely abolished by 500-fold excess of unlabeled GDNF (lane 2). No proteins were precipitated by monoclonal anti-neurofilament antibodies (lane 3) or by Protein A-Sepharose only (lane 4). No cross linked complexes were obtained from COS cells (not shown). Since *c-ret*

25   proto-oncogene is a glycoprotein, ^{125}I-labeled NB2/a cell extracts were also immunoprecipitated with wheat germ agglutinin. Again, proteins of 170 and 190 kD were obtained (lane 5).

To establish further that GDNF specifically binds c-RET, the mouse *c-ret* cDNA

was cloned into the mammalian expression vector PBK-CMV and transiently expressed in monkey COS cells. Mouse c-ret cDNA (Pachnis, V., Mankoo, B, & Costantini, F. *Development*, 119, 1005-1017 (1993)) in pbluescript SK' (Stratagene) was cleaved with *SacII* and EcoRV and cloned into *SacII and SmaI* site of pBK-CNV vector (Strategene).

5   COS cells were transiently transfected with c-ret cDNA or with empty plasmid by electroporation (Bio Rad) with - 30 % efficiency by fluorescence of cotransfected Red Shift Green Fluorescent Protein in PEF-BOS vector. 48 hours later, 10×10^6 transfected COS cells or $3-5 \times 10^6$ parental COS cells or NB2/a cells were treated With ^{125}I-GDNF, cross linked and analysed as specified in legends of Fig. 15 and Fig. 16.

10   First, the expression of c-RET protein was examined by Western blotting. c-ret-transfected COS cells (Fig. 18a) and NB2/a cells (not shown) expressed detectable amounts of the c-RET protein, whereas no c-RET protein was detected in mock-transfected (with PBK-CMV plasmid) COS cells (Fig. 18a). PC12 cells also express c-RET protein, albeit at considerably lower level than NB2/a cells or c-ret-transfected COS

15   cells (not shown). COS cells, transiently expressing mouse c-ret proto-oncogene were incubated with ^{125}I-GDNF. As shown in Fig. 17b, those cells bound GDNF, and binding of ^{125}I-GDNF can be competed with excess of unlabeled GDNF. In contrast, no significant binding-of GDNF was observed in mock-transfected COS cells.

20   **Example 16**

Phosphorylation of tyrosine residues

       10×10^6 transfected COS cells (48 hr after transfection) were treated with 50 ng/ml of GDNF (Preprotech EC Ldt.) for 5 minutes in serum-free DMEM, or not treated, and then quickly washed with the same medium. NB2/a cells were similarly

25   treated (results not shown). c-RET proteins were immunoprecipitated from detergent extracts by cocktail of monoclonal (Lo, L. & Anderson, D. *J. Neuron, 15,* 527-539 (1995) and polyclonal (Santa Cruz) anti-c-ret antibodies, separated by 7% SDS-PAGE, transferred to nitrocellulose, probed by anti-c-ret antibodies (Santa Cruz), stripped and

reprobed by anti-phosphotyrosine antibodies (Sigma).

This treatment resulted in significant increase in tyrosine phosphorylation of 190 kD cRET proto-oncogene, the 170 kD form being less prominently phosphorylated (Fig. 18b). In both cell lines, relatively high c-RET phosphorylation was detected also in the

5   absence of GDNF (Fig. 18), most probably via endogenous GDNF secreted by these cells and/or ligand-independent receptor dimerization.

Example 17

10   **^{125}1-GDNF binds to c-ret-positive enteric neurons**

^{125}I-GDNF was bound to developing rat tissue explants *in situ*. *In situ* binding of human ^{125}I-GDNF (PeproTech. EC Ltd.), iodinated by Chloramine T Method, was carried out essentially as described (Partanen and Thesleff, 1987). Briefly, explants of E15 rat gut were incubated with 10 ng/ml of ^{125}I-GDNF in Eagle's minimal essential

15   medium on the Nuclepore filter (Costar) for 90 min at room temperature. 250-fold excess of unlabeled GDNF was applied as a competitor to control explants. After careful washing, the explants were fixed with 3.5% paraformaldehyde in PBS, sectioned and exposed to NTB-2 emulsion (Kodak).

The gastrointestinal tract was chosen as it strongly expresses GDNF mRNA

20   (Suvanto *et al.*, 1996); Figure 19 a and b) and c-RET-positive neurons are absent in the gastrointestinal tract in *c-ret*-deficient mice (Schuchart et al., 1994; Durbec et al., 1996). ^{125}I-GDNF binds to a group of cells within the muscle layer of embryonic day (E)15 rat gut (Figure 19 c and d). This binding was specific as it was totally competed with 250-fold excess of unlabeled GDNF (Figure 19h). The cells that bind GDNF were the enteric

25   neurons of the myenteric plexus, as revealed by peripherin immunoreactivity (Figure 19f).

Moreover, these neurons also expressed *c-ret* mRNA, as demonstrated by *in situ* hybridization (Figure 19e). Cloning of the GDNF cDNA and *in situ* hybridization with GDNF probe was performed exactly as described (Suvanto *et al.*, 1996). A 646 bp long

fragment of mouse *c-ret* cDNA (Pachnis et al., 1993) covering the 3'-region of the

shorter form (Takahashi et al., 1988) of *c-ret* was cloned into *NotI-XhoI* site of pBSK+

vector (Stratagene). cRNAs in antisense and sense orientation were labeled with

digoxigenin-UTP (Boehringer-Mannheim), hybridized to cryosections through E15 rat gut

5   and visualized with alkaline phosphatase-conjugated anti-digoxigenin antibodies according

to manufacturers instructions. In both cases, only background labeling was obtained with

hybridization of corresponding probes in sense orientation (Figure 19g). Polyclonal anti-

peripherin antibodies (Bio-Rad) were applied to cryosections of E15 rat gut at a dilution

of 1:100 for 1 hr and visualized by FITC-conjugated secondary antibodies (Jackson).

10   Thus, GDNF specifically binds to c-RET-expressing enteric neurons of developing rat.

Example 18

Affinity-cross linking of GDNF to c-RET

PC12 cells and NB2/a cells were washed three times with serum-free RPMI-1640

15   or DMEM, respectively, plated on uncoated (NB2/a cells) or collagen-coated (PC12 cells)

dishes (5000-6000 cells per dish in triplicate) in the presence or absence of 50 ng/ml of

GDNF (PeproTech EC Ltd.), and the number of cells microscopically counted after 48 h.

For c-RET expression in transfected cells, the shorter form (Takahashi el al.,

1988) of human wild-type *c-ret* cDNA was subcloned in pcDNA3 (Invitrogen). 3T3

20   fibroblasts were stably transfected with c-ret expression plasmid or with empty vector

(mock-transfected cells) and positive cells lines selected with G418.

Transient transfection of trkC 3T3 fibroblasts (Ip et al. (1993) *Neuron*, 10, 137-

149) with human *c-ret* cDNA in pcDNA3 vector or with empty vector was performed by

the lipofectin method (Gibco-BRI.). c-ret and mock-transfected cells (10.000 - 15.000

25   cells per well) in five parallels were treated with rat GDNF (Trupp et al. (1995) *J. Cell.

Biol.* 130, 137-148) at indicated concentrations for five days. NT-3 was used as positive

control at 30 ng/ml. Cell number was quantified by measurement of acid phosphatase

activity using Abacus™ Cell Proliferation Kit (Clontech).

-54-

3-5 x 10^6 PC12 cells, NB2/a cells, COS cells or c-ret-3T3 as well as mock-3T3 cells or mechanically dissociated cells from two F20 or from 17 E15 rat kidneys were incubated with 10 ng/ml of ^{125}I-GDNF (human GDNF from PeproTech EC Ltd. or rat GDNF from C. F. Ibanez) (Trupp et al., 1995), iodinated by Chloramine T method, for

5 1 hour on ice. 250-fold excess of unlabeled GDNF (PeproTech EC) or TGF-β1 (kindly provided by Dr. M. Laiho) was applied to control sample. ^{125}I-GDNF was then crosslinked to the cells with 30 mM of ethyl-dimethylaminopropyl carbodiimide (EDAC) (Pierce) for 30 minutes on ice. Detergent lysates of the cells were immunoprecipitated with polyclonal anti-GDNF antibodies (Santa Cruz) or with the cocktail of monoclonal

10 (kindly provided by Dr. D. Anderson, Lo and Anderson, 1995) and polyclonal (Santa Cruz) anti-c-RET antibodies to neurofilament proteins (a gift of Dr. I. Virtanen) were used as control antibodies. The precipitates were collected by Protein A-Sepharose (Pharmacia) or by WGA-agarose (a gift from Dr. O Renkonen), separated on 7% SDS-PACE, and visualized with a Phosphorimager SI (Molecular DynanLics).

15 First, ^{125}I-GDNF was cross linked to PC12 cells, NB2/a cells and COS cells with ethyl-dimethyiaminopropyl carbodiimide (EDAC), and the complexes were precipitated with anti-GDNF antibodies. As shown on Figure 20a, complexes with molecular weight of 190 kD and 170 kD were obtained from PC12 and NB2/A cells, but not from COS cells. The molecular weights of the cross linked proteins (minus GDNF monomer of

20 ~25K) correspond to those of c-RET, (140 kD and 160 kD, representing partially and fully glycosylated isoforms of c-RET, respectively) (Takahashi et al., 1988).

Next, ^{125}I-GDNF was cross linked to PC12 and NB2/a cells by EDAC and immunoprecipitated formed complexes with anti-c-RET antibodies. The bands with molecular weight of 190 kD were obtained from both cell lines (Figure 20 a and b).

25 Formation of the complexes was abolished by 500-fold excess of unlabeled GDNF. The reason why both fully and partially glycosylated forms of c-RET were precipitated by anti-GDNF antibodies, but only the larger isoform by anti-c-RET antibodies, is unclear. The same complexes, although much weaker, were also obtained when

dithiobis(succinimidylpropionate) was used as a crosslinker (data not shown).

The EDAC-crosslink approach was also used to reveal GDNF-c-RET complexes from E15 embryonic kidney cells, where c-ret mRNA is strongly expressed in the tips of growing ureter branches. With both anti-GDNF and anti-c-RET antibodies, a band of

5   190 kD was obtained (Figure 20 a and b) that was competed with excess of unlabeled GDNF. Thus, only the fully glycosylated form of c-RET is expressed in embryonic kidney cells.

Cross linked ^{125}I-GDNF-c-RET complexes from the cells ectopically expressing *c-ret* were also demonstrated. 3T3 cells were transfected with *c-ret* cDNA or with empty

10   plasmid, and established stable transfected cell lines (*c-ret*-3T3 cells or mock-3T3 cells). Cross linking of ^{125}I-GDNF to these cells followed by anti-RET-precipitation revealed a 190 kD band that was abolished with 250-fold excess of unlabeled GDNF (Figure 20b). As GDNF is a distant member of TGF-β family, we also used a 250-fold excess of TGF-β1 as a competitor. No competition was observed with TGF-β1 (Figure 20b) Taken

15   together, these data show that GDNF directly and specifically binds to c-RET.

Example 19

GDNF specifically increases tyrosine phosphorylation of c-RET

20   *c-ret*-3T3 cells and mock-3T3 cells were treated with GDNF and the proteins from these cells were immunoprecipitated with anti-c-RET antibodies. The precipitated proteins were then analyzed by Western blotting with anti-phosphotyrosine antibodies.

10 x 10⁶ c-ret-3T3 cells were treated with different doses of GDNF (PeproTech LC Ltd. or from C. F. Ibanez) (Trupp *et al.*, 1995) for 5 min, or with 50 ng/ml of

25   GDNF for indicated times in serum-free Dulbecco's modified Eagle's medium containing 1 mM Na_3VO_4 and then quickly washed with the same medium. c-RET proteins were immunoprecipitated from detergent extracts, containing 1 mM Na_3VO_4 by cocktail of monoclonal (Lo, L. and Anderson, D.J. (1995) *Neuron*, 15 527-539) and polyclonal

(Santa Cruz) anti-c-RET antibodies, separated by 7% SDS-PAGE and transfected to nitrocellulose which were probed by anti-phosphotyrosine antibodies to nitrocellulose with was probed by anti-phosphotyrosine antibodies PY20 (Transduction Laboratories), then stripped and reprobed by anti-c-RET antibodies (Santa Cruz).

5   As shown on Figure 21a, a short treatment of c-ret-3T3 cells with GDNF dose-dependently (beginning at 25 ng/ml) increased tyrosine phosphorylation of the 160 kD c-RET isoform, whereas the phosphorylation of the 140 kD isoform remained unchanged. An increase in c-RET phosphorylation was evident at 25 ng/ml of GDNF and above it. No c-RET proteins were detected in mock-3T3 cells. c-ret-3T3 cells were also treated

10   with GDNF (50 ng/ml) for different times. An increase in c-RET tyrosine phosphorylation was evident after 5 minutes of treatment and continued at least for one hour (Figure 21b). With prolonged exposition, the increase in phosphorylation of lower c-RET isoform also became evident. A basal level of c-RET phosphorylation was detected in the absence of GDNF, possibly via a ligand-independent dimerization of that

15   receptor. To reveal the amounts of c-RET protein in these experiments, the filters were stripped from antibodies and reprobed with anti-c-RET antibodies. The level of c-RET protein was not changed by GDNF treatment in c-ret-3T3 cells (Figure 21a and b, lower panels). The finding that GDNF specifically activates c-RET indicates that c-RET is a signaling receptor for GDNF.

20

Example 20

c-RET expression confers GDNF-responsiveness to 3T3 cells

         Mouse 3T3 fibroblast cell line expressing trkC (trkC-3T3) (Ip et al, 1993) were transiently transformed with c-ret expression plasmid. trkC-3T3 cells die within 2-3 days

25   in serum-free medium in the absence of trkC ligand neurotrophin-3 (NT-3) (Ip et al., 1993) and do not express detectable amounts of c-ret. GDNF dose-dependently increased the number of c-ret-transfected but not of mock-transfected trkC-3T3 cells (Figure 22), which was comparable to the response elicited by NT-3. Whether this is a proliferative

or survival-promoting response could not be distinguished based upon the data. Thus, introduction of c-RET to GDNF-nonresponsive cells is sufficient to bring about the biological response to GDNF.

5

Example 21
Isolation of GDNF receptor

L6 myoblast cells were lysed with 1% NP40 and cell lysates were fractionated by anionic exchange on a Q-Sepharose column. Fractions eluted at different ionic

10 strength were dialyzed and assayed for binding to GDNF immoliblized on a chip in a Biacore device (Pharmacia). A distinct binding component was detected in a fraction of L6 cell lystaes (Figure 23a). In the Figure, solid bars indicate total protein (as absorbance at 280 nm); hatched bars indicate GDNF finding (in resonance units). This fraction was not particularly rich in protein, indicating a substantial purification over the

15 total protein mixture. The equivalent fraction of a COS cell lysate did not show binding under the same conditions (data not shown).

Further purification of the GDNF binding activity in the first 1M salt fraction was obtained after hydrophobic interaction chromatography (Figure 23b). The data represents the ratio between GDNF binding (in resonance units) and protein concentration (OD at

20 280 nm). Fractions were eluted with a step-wise gradient of ammonium sulfate.

Alternatively, purification may be effected by cross linking GDNF to cells in the presence of tracer amounts of radiolabeled ligand, and ligand/receptor complexes can by fractionated through ion exchange chromatography followed by hydrophobic interaction chromatography and SDS/PAGE. Bands corresponding to the molecular weights of

25 GDNF-receptor complexes can by excised, dissociated, and then sequenced by mass spectrometry or Edman degradation, depending upon the yield of recovery.

Example 22

A novel GDNF-binding protein in brain

By ligand blotting, we have identified another GDNF-binding protein from total brain extract. We bound ^{125}I-GDNF to the filters carrying protein from the total extracts of brain and liver (a ligand blot assay). A major band with MW of about 50 kD was obtained from brain extract, but not from liver (Figure 24). This binding is specific as ^{125}I-GDNF did not bind to other proteins from total lysates, it is not found in liver lysates (nor in some other tissues), and it can be competed with excess of unlabeled GDNF.

Binding of ^{125}I-GDNF to c-RET was not revealed in the ligand blots. The reason for this may be the very low share of c-RET in total brain extract. Alternatively, by analogy with other receptors for GDNF, c-RET might not bind GDNF directly, but might first bind to another nonsignalling receptor that thereafter presents the ligand to c-RET, a signaling receptor. A 50 kD GDNF-binding protein is a good candidate for the putative presenting receptor.

Example 23

Protocol for isolating novel signaling receptors for GDNF

In the absence of serum, 3T3 fibroblasts can be made dependent on a given exogenous growth factor provided appropriate receptors are expressed on the cell surface. An expression library can be made using RN33B cDNA, which can then be transfected into 3T3 fibroblasts by procedures well known in the art (Maniatis et al., *supra*). Stable transfectants can be selected in serum-free media supplemented with GDNF. Fibroblast clones that express signaling GDNF receptors will selectively grow in the presence of GDNF in serum-free media. The selection step may allow detection of even very reare clones due to their differential growth advantage. Further analysis of the recovered clones in media with or without GDNF would help to distinguish GDNF-dependent from GDNF-independent survival of clones.

Example 24

Cloning and Sequencing of GDNFR-β

   Rat:

      A BLAST search of the publicly available genomic database at NCBI (National

5   Center for Biotechnology Information) was performed using the amino acid sequence for

GDNFR-α as reported in Jing et al. (1996) *Cell* 85:1113-1124 (hereby incorporated by

reference). The search revealed several human ESTs with homology to the GDNFR-α .

Primers were designed from these sequences and a partial cDNA clone was amplified

from human fetal brain RNA. The upstream PCR primer was

10   5'ATGGATCCGCAACCTGAATGACAACTGC3' [SEQ ID NO: 3]. The downstream

PCR primer was 5'CCGAATTCAGTTGGGCTTCTCCTTGTC3' [SEQ ID NO: 4]. This

cDNA clone was used to screen a rat brain cDNA library from which a nearly complete

cDNA was isolated. Additional PCRs were used to clone the most 5' of this cDNA.

The complete cDNA sequence is depicted in Figure 32 (SEQ ID NO:5).   The amino

15   acid sequence of the complete cDNA clone was determined and is depicted in Figure 25.

It is 464 amino acids long. A putative signal peptide and a GPI anchoring motif were

identified in the N-terminal 15 and C-terminal 17 amino acid residues, respectively.

   Human:

20   Rat GDNFR-α sequence (6 GenBank ™ accession number U59486) was used to

screen the human EST database and 8 human sequences from 6 different clones (GenBank

™ accession numbers H12981, H05619, R02135, R02249, T03342, W73681, W73633

and Z43761) with high similarity (identity > 70% over 200 bp) were identified. Full

sequence analysis of three EST clones revealed a 3'-terminal consensus sequence which

25   we designated human EST GDNFR-β cDNA (nucleotides 469-1490 in Fig. 33).

      To obtain complete cDNA, human EST GDNFR-β cDNA was used for screening

an adult rat hippocampus cDNA library (λZap, Stratagene). Two identical clones out of

one million contained a 2002 base pair long sequence with 91% identity to the probe.

With a forward primer designed from rat GDNFR-β sequence, the 5' end of human

GDNFR-β was amplified by PCR from human total brain cDNA. Human total RNA was

extracted by standard methods and reverse transcribed in a random-primed reaction as

described in Superscript II (Life Technologies) protocol. The human GDNFR-β gene

5   was amplified from cDNA under the following PCR conditions: dNTP's in 200 μM

concentration and primers (forward) 5'-ATGATCTTGGCAAACGCCTTCTG-3 [SEQ ID

NO: 6] and (reverse) 5'-TTGCAGTTGTCATTCAGGTTGC-3' [SEQ ID NO: 7] in 1 μM

concentration, approximately 5 ng of human brain cDNA, 1 u of Dynazyme (Finnzymes)

Taq polymerase in 50μl. The 30 cycles after initial 5 minutes at 94°C consisted of 30 s

10   at 94°, 30 s at 57°C and 1 min at 72°C with a final 5 minute extension at 72°C. The

PCR fragments were cloned into pGEM-T vector (Promega) and four different clones

were sequenced. With several primer pairs complete GDNFR-β cDNA sequence was

amplified by PCR from the same human brain cDNA. This sequence was identical to the

EST-derived sequence. The overlapping inserts of EST and PCR fragments were

15   combined and cloned to get the contig of the full-length human cDNA. The human

GDNFR-β cDNA sequence (GenBank ™ accession number U93703) contains a 1395 base

pair long open reading frame (Fig. 33).

At amino acid level, human and rat GDNFR-β orthologues were 96% identical.

The predicted 47kD (unglycosylated) mature protein, consists of 464 amino acids that

20   are 48% identical and up to 63% similar to the published sequence of human and rat

GDNFR-α proteins (Jing et al., *supra*) (Fig. 26). Alignment was performed using MAP

(multiple alignment program). (Huang, X, *Comp. Appl. BioSci.*, 10: 227-235, 1994.

The putative signal sequence was predicted according to von Heijne, *Nucl. Acids. Res.*,

14:4683-4699, 1986). The amino acid sequence of GDNFR-β has a putative signal

25   sequence, three N-glycosylation sites, and a putative GPI-anchor site similar to GDNFR-

α. Completely conserved cystein residues and strong overall resemblance to GDNFR-α

predict high similarity in the spatial structures of the GDNF-receptors α and β (Fig. 26).

Amino acid identity as used herein means the same amino acid residue. Amino

acid similarity as used herein means the same or chemically similar amino acid residue (e.g., Lys is identical to Lys, but is similar to Arg).

Example 25

5   **GDNFR-α and GDNFR-β expression**

Expression of mRNA for GDNFR-α and GDNFR-β was investigated by RNAse protection analysis, as disclosed in Example 4 above, in cells known to express GDNF receptors as determined from chemical cross linking studies. GDNFR-α mRNA was found in RN33B and MN1 cells (Figure 27). High levels of GDNFR-β mRNA were

10 detected in RN33B cells. Lower levels were found in post-natal (P1) kidney and in MN1 cells. (See Figure 28.) No GDNFR-α or GDNFR-β mRNA could be detected in L6 cells (Figures 27 and 28).

The mRNA expression patterns of GDNFR-α and GDNFR-β mRNAs were also determined and compared in E17 rat embryo by *in situ* hybridization. *In situ*

15 hybridization on E17 rat sections was performed exactly as described previously (Suranto et al. *Eur. J. Neurosc.*, 8:816-822, 1996; Wilkinson et al. in Post Implantation Mammalian Embryos a Practical Approach, IRL Press, Oxford, Copp., A.J. and Cockroft, D.L., eds. pp. 151-171, 1990, both incorporated herein by reference). The antisense cRNA probes for rat GDNFR-α and rat GDNFR-β covered nucleotides 294-

20 1039 of GenBank™ sequence U59486 and nucleotides 1231-1394 of the rat GDNFR-β sequence, respectively. In several organs, GDNFR-α and GDNFR-β mRNAs showed distinct, non-overlapping distributions. Strong GDNFR-β mRNA expression was seen in the capsule and cortex of adrenal gland (Fig. 37B), whereas GDNFR-α mRNA was detected in adrenal medulla (Fig. 37C). In kidney, GDNFR-α transcripts were seen in

25 the tips of ureter bud, condensed mesenchyme and early epithelial tubules (Fig. 37F), whereas GDNFR-β mRNA was present in undifferentiated nephrogenic mesenchyme and in the muscle wall of renal pelvis (Fig. 37E). Strong expression of GDNFR-α mRNA was present in the muscle and nervous layers along gastrointestinal tract (Fig. 37I). In

stomach, GDNFR-β mRNA was moderately expressed in nervous layers (not shown),

while in enteric plexus of small intestine only low amounts of transcripts were detected

(Fig. 37H). In embryonic (E)17 rat nervous system, GDNFR-α mRNA was abundantly

expressed in spinal cord, especially in ventral motoneurons (Fig. 37J). Also GDNFR-β

5   mRNA was present in many areas of spinal cord including ventral motoneurons (Fig. 5J,

K), though the levels were moderate. In dorsal root and trigeminal ganglia, GDNFR-β

mRNA expression was restricted to subpopulations of neuronal cells (Fig 37K,M,N),

resembling Ret mRNA distribution (Pachnis et al., *Development*, 119:1005-1017, 1993).

In contrast, varying amounts of GDNFR-α transcripts could be detected throughout the

10   ganglia (Fig 37J,L,M,O) and also in the cells covering trigeminal and spinal nerves (Fig.

37 M,O), suggesting expression in both neuronal and glial cells.

Example 26

15   **Affinity Cross Linking of GDNF to GDNFR-α and GDNFR-β**

COS cells were transfected with GDNFR-α, GDNFR-β, c-RET, and a control

plasmid and affinity cross linking studies were performed as disclosed in Example 15

above. The results are depicted in Figure 29. COS cells transfected with a control

plasmid did not bind GDNF (lanes 1 and 2). Transfection with a GDNFR-α cDNA

20   followed by incubation with ^{125}I-GDNF generated a GDNF binding complex with an

approximate molecular weight of 70kD (lane 3). The formation of this complex could be

prevented by incubation with cold GDNF (lane 4).

Transfection with a GDNFR-β cDNA generated a GDNF binding complex with an

approximate molecular weight of 100 kD (lane 5). The formation of this complex could

25   also be prevented by incubation with cold GDNF (lane 6). Both GDNFR-α and GDNFR-

β facilitated specific cross linking of ^{125}I-GDNF to c-RET (lanes 7 to 10). COS cells

transfected with a c-Ret cDNA alone did not bind GDNF (lanes 11 and 12). Molecular

weight markers and the positions of the different GDNF receptor complexes are

indicated.

Example 27

5   **Comparison with Complexes from Cross Linking Studies with RN33B Cells**

When compared side-by-side, the complex of GDNFR-α and GDNF formed in the transfected COS cells ran at the same molecular weight as the 70 kD complex detected in RN33B cells after GDNF cross linking with EDAC performed as disclosed in Example 2 (Figure 30, compare lanes 1 and 2). The complex of GDNFR-β and GDNF formed in

10   the transfected COS cells ran at the same molecular weight as the 100 kD complex detected in RN33B cells (compare lanes 1 and 3). The identity of the receptor component in the 155 kD binding complex is unknown, but it is formed by binding to the 135k GDNF-binding protein identified above.

15   **Example 28**

Phosphorylation of cRET

Upon transfection into COS cells, cRET is highly phosphorylated on tyrosine residues independent of the presence or absence of GDNF (Figure 31, upper panel). COS cells were transfected with 4 μg of c-Ret plasmid and 16 μg control plasmid.

20   Transfected COS cells were exposed to different concentrations of GDNF using the procedure disclosed in Example 16, and cytoplasmic cell lysates were immunoprecipitated with anti-c-RET antibodies. Precipitated c-RET receptors were analyzed for tyrosine phosphorylation by Western blotting using anti-P-tyrosine monoclonal antibodies.

Co-transfection with plasmids encoding GDNFR-α or GDNFR-β (in place of the

25   control plasmid), although allowing for ligand stimulation of receptor tyrosine phosphorylation in a dose-dependent manner, resulted in a dramatic attenuation of the steady-state levels of tyrosine phosphorylation of c-RET, i.e., in the absence of GDNF (Figure 31, upper panel). Reprobing of the same blot with anti-c-RET antibodies

revealed that all lanes contained equal amounts of c-RET (data not shown).

When COS-7 cells were transiently transfected with cDNAs encoding GDNF receptors in different combinations and treated with GDNF (100 ng/ml), it induced phosphorylation of the 170 kD formm of R*et* only in the presence of either GDNFR-α or

5   GDNFR-β (Fig. 36, lanes 1-4), but not without these receptors (Fig. 36, lanes 5-6). The 170kD from of R*et* represents the fully glycosylated mature receptor on the plasma membrane (Takahashi et al. *Oncogene*, 3:571-578, 1988). In control cells cotransfected with GDNFR-α and GDNFR-β but without R*et*, no tyrosine phosphorylated proteins were precipitated by anti-R*et* antibodies (Fig. 36, lanes 7-8). Identical results were gained

10   when a GDNFR-β construct lacking GPI-anchor was used (data not shown). Tyrosine phoshorylation of immature, partially glycosylated 150 kD form of R*et* was not increased by GDNF treatment. The nature of the 85 kD bands in GDNFR-α transfected cells is not known. The major 50kD band in all lanes is the heavy chain of IgG.

Human full-length GDNFR-β cDNA was cloned into pCDNA3 (Invitrogen) and

15   pBk-CMV (Stratagene) mammalian expression vectors. Rat GDNF-β cDNA was cloned into the same expression vectors. In one rat construct, the 3' end of human GDNFR-β cDNA was added using a unique BclI restriction site and in another construct, an artificial stop codon was inserted instead of GPI-tail, producing an apparent soluble form of rat GDNFR-β.

20   COS-7 cells (5 x 10^6 cells per experimental point) were cotransfected by electroporation (Bio-Rad) with cDNAs (5 μg each) of R*et* and GDNFR-α, R*et* and GDNFR-β, GDNFR-α, and GDNFR-β, or with cDNA of R*et* alone and cultured for 48 hours. Cellular phosphatases were inhibited by 1 mM Na_3, VO^4 for one hour, the cells were treated with 100 ng/ml of GDNF (Promega or PeproTech Ltd) for 30 minutes and

25   lysed in Tris-balanced saline. pH 7.5, containing 2 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1% Triton x-100 and protease inhibitors. Proteins immunoprecipitated by anit-RET antibodies (Santa Cruz) were analysed by Western blotting with anti-phosphotyrosine antibodies PY20 (Transduction Laboratories). In experiments with the

secreted form of GDNF-β lacking an GPI anchor, the cells were not washed before GDNF treatment.

Neuro-2A cells express c-RET endogenously, but show low levels of constitutive receptor tyrosine phosphorylation (Figure 31, lower panel). However, transfection of the

5   Neuro-2A cells using procedures disclosed above resulted in stimulation of tyrosine phosphorylation of the c-RET receptor in the presence of GDNF in a dose-dependent manner (Figure 31, lower panel).

Example 29

10 **Tissue distribution of GDNFR-β**

The tissue distribution of human GDNFR-β was studied by Northern hybridisation of mRNA extracted from different adult and fetal tissues. The expression of GDNFR-β mRNA was abundant in adult brain, intestine and placenta, as well as in fetal brain, lung and kidney. Two major transcripts of 2.9 and 3.5 kb were visible in all tissues, and

15 additional transcripts of 7.5 kb in placenta and 1.4 kb in testis (Fig. 34) were found.

For Northern Hybridization, 100 ng of the human EST GDNFR-β insert was labeled with ^{32}P-dCTP (Amersham) by Prime-a-Gene-Kit (Promega). The specific activity of the final probe was 2 x 10^7 cpm/μg and the hybridization of Human and Human Fetal Multiple Tissue Northern Blot filters (Clontech) was performed in

20 ExpressHyb solution at 65° for 2 hours. The filters were washed two times for 30 minutes at 50°C in 2x saline sodium citrate (SSC) + 0.1% SDS and 0.1% SSC + 0.1% SDS and then analysed by phosphoimager (Fuji BAS 1500). As a control, the same filters were hybridized with human β-actin probe (Clontech).

25 **Example 30**

GDNFR-β Locus

The chromosomal locus of GDNFR-β was assigned to human chromosome 8p21-22 by fluorescent *in situ* hybridization (FISH) with the 1.49 kb human GDNFR-β cDNA

probe. In addition, the locus for mouse GDNFR-β gene was assigned to the mouse

chromosome 14D3-E1 that corresponds to the human locus 8p21-22 (Fig. 35).

Human peripheral blood lymphocytes were cultured and the cell culture from a

mouse fetal tissue was established according to standard protocols (Fresney, I.R., Culture

5   of Animal Cells, Manual of Basic Techniques, Allan R. Lisa, Inc. New York, 1983)

and used as a source of metaphases chromosomes. Both human lymphocytes and mouse

monolayer cells were treated with 5-bromeoxyuridine at early replicating phase to induce

a banding pattern (Takanashi, et al. *Hum. Gene*, 86:14-16, 1990; Lemieux et al., *Cell

Gene*, 59:311-312, 1992). The slides were stained with Hoechst 33258 (1μg/ml) and

10   exposed to UV-light. (302 nm) for 30 min. The probes were labeled by a nick

translation kit (BRL) with biotin-11-dUTP (Sigma) and the FISH procedure was carried

out in 50% formamide, 10% dextran sulphate in 2xSSC as described earlier (Lichter et

al., *Proc. Natl. Acad. Sci. USA*, 85:9664-9665, 1988; Pinkel et al, *Proc. Natl. Acad.

Sci., USA*, 83:2934-2938, 1986, both incorporated herein by reference). Repetitive

15   sequences were suppressed with 10-fold excess of Cot-1-DNA (BRL) and after overnight

incubation unspecific hybridization signals were eliminated by washing the slides with

50% formamide/ 2x SSC, 2x SSC and 0.5x SSC at 45°C. Specific hybridization signals

were visualized using FITC-conjugated avidin (Vecotr Laboratories) and slides were

counterstained with 4'-6'-diamino-2-phenylindole (25 ng/ml). The image analysis for

20   acquisition, display and quantification of hybridization signals was performed with a PXL

camera (Photometrics) attached to a PowerMac 7100/AV workstation. IPLab software

controls the camera operation, image acquisition and Ludl wheel (Heiskanen, et al.

Genet. Anal. Biol. Eng., 12:179-184, 1996). The probe for human GDNFR-β gene was

1490 bp long cDNA and the hybridization showed specific double spot signal in 30 out of

25   100 metaphase chromosomes that were identified based on their G-banding pattern. The

hybridization signal of the 10 kb genomic mouse probe showed specific localization in 27

out of 30 mouse metaphase chromosomes (Cowell, et al., *Chromosome*, 89:294-320,

1984).

All references cited herein are hereby incorporated by reference in their entireties.